

Spectrin and its interacting partners in nuclear structure and function

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Impact statement

The nucleoskeleton is critical for maintaining the architecture and functional integrity of the nucleus. Nonerythroid α -spectrin (α IISp) is an essential nucleoskeletal protein; however, its interactions with other structural and non-structural nuclear proteins and its functional importance in the nucleus have only begun to be explored. This review addresses these issues. It describes α IISp's association with DNA repair proteins and at least one proposed mechanism of action for its role in DNA repair. Specific interactions of α IISp with other nucleoskeletal proteins as well as its important role in the biomechanical properties of the nucleus are reviewed. The consequences of loss of α IISp, in disorders such as Fanconi anemia, are examined, providing insights into the profound impact of this loss on critical processes known to be abnormal in FA, such as development, carcinogenesis, cancer progression and cellular functions dependent upon α IISp's interactions with other nucleoskeletal proteins.

Abstract

Nonerythroid α IISp is a structural protein whose roles in the nucleus have just begun to be explored. α IISp is an important component of the nucleoskeleton and has both structural and non-structural functions. Its best known role is in repair of DNA ICLs both in genomic and telomeric DNA. α IISp aids in the recruitment of repair proteins to sites of damage and a proposed mechanism of action is presented. It interacts with a number of different groups of proteins in the nucleus, indicating it has roles in additional cellular functions. α IISp, in its structural role, associates/co-purifies with proteins important in maintaining the architecture and mechanical properties of the nucleus such as lamin, emerin, actin, protein 4.1, nuclear myosin, and SUN proteins. It is important for the resilience and elasticity of the nucleus. Thus, α IISp's role in cellular functions is complex due to its structural as well as non-structural roles and understanding the consequences of a loss or deficiency of α IISp in the nucleus is a significant challenge. In the bone marrow failure disorder, Fanconi anemia, there is a deficiency in α IISp and, among other characteristics, there is defective DNA repair, chromosome instability, and congenital abnormalities. One may speculate that a deficiency in α IISp plays an important role not only in the DNA repair defect but also in the congenital anomalies observed in Fanconi anemia, particularly since α IISp has been shown to be important in embryonic development in a mouse model. The dual roles of α IISp in the nucleus in both structural and

non-structural functions make this an extremely important protein which needs to be investigated further. Such investigations should help unravel the complexities of α IISp's interactions with other nuclear proteins and enhance our understanding of the pathogenesis of disorders, such as Fanconi anemia, in which there is a deficiency in α IISp.

Keywords: Nonerythroid alpha spectrin, DNA interstrand cross-link repair, telomere function and dysfunction, Fanconi anemia, nucleoskeleton, peripheral nucleoskeletal proteins

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Introduction

The nucleus houses not only the genome but also a complex group of proteins that associates with the nucleoskeleton and which contributes to the structural and functional complexity of the nucleus. This network of proteins is essential for the structural integrity of the nucleus as well as for proper regulation of DNA metabolism, DNA replication

and transcription and DNA repair.^{1–9} Among these proteins is nonerythroid alpha spectrin (α IISp).^{7,10–14} In the nucleus, α IISp is best known for its role in DNA repair and chromosome stability.^{12–19} It directly interacts with damaged DNA and with proteins involved in DNA repair. In addition, it associates with structural proteins in the nucleoskeleton such as lamin, emerin, actin, protein 4.1,

and nuclear myosin^{7,19–21} and through these interactions is thought to make an important contribution to the structural integrity of the nucleus as well as its elasticity.^{7,22}

α IISp consists of an extended array of triple α -helical repeats which contributes to the elasticity of the nucleoskeleton with which it associates.^{21,23–26} It contains a SH3 domain, which is a site that plays an important role in protein–protein interactions and assembly of protein networks involved in intracellular signaling, signal transduction, and protein synthesis.^{27–31} We have shown that the Fanconi anemia protein, FANCG, binds directly to the SH3 domain of α IISp and have hypothesized that this binding is important in maintaining the stability of α IISp in the nucleus.³² We have demonstrated that α IISp is important in repair of DNA interstrand cross-links (ICLs) in both genomic (non-telomeric) and telomeric DNA and plays a critical role in maintaining genomic stability after DNA damage.^{12–19} Based on these studies, we have proposed that α IISp acts as a scaffold to recruit DNA repair proteins to sites of damage.^{12,18}

In addition to α IISp's interaction with DNA repair proteins, it also interacts with a number of other functionally important groups of proteins in the nucleus (Figure 1). These include structural proteins, such as lamin, emerin, actin, nuclear myosin, proteins important in chromatin remodeling, Fanconi anemia (FA) proteins, and proteins involved in transcription and RNA processing (Figure 1).^{7,19–21} Thus, α IISp's interaction with a diverse group of proteins in the nucleus indicates that it has multiple roles in various nuclear processes (Figure 1). This review will concentrate on the functional importance of the interaction of α IISp in the nucleus of human cells with proteins involved in DNA repair, particularly those involved in the repair of DNA ICLs, and with structural proteins associated with the nucleoskeleton. This is an area of expanding research as new proteins interacting with α IISp are identified and new roles for these interactions are demonstrated.

α IISp interacts with DNA repair proteins and is linked to DNA repair

α IISp has long been recognized as an important component of the cytoskeletal network.^{23–25} It interacts with β -spectrins in the cytoplasm to form a tetramer of α/β spectrin which plays a role in cell architecture and structural support of the plasma membrane, trafficking of proteins, vesicles and organelles, signal transduction, synaptic transmission in neurons, cell division and proliferation, cell adhesion, and cell development and morphogenesis (Figure 1).^{23–25,33–39} α IISp is also present in the nucleus but a similar association with β -spectrins has not, as yet, been demonstrated.¹⁹

In the nucleus, α IISp has been shown to play an important role in DNA repair.^{12–19} It interacts with proteins involved in DNA interstrand cross-link repair, nucleotide excision repair (NER), homologous recombination repair (HRR), and non-homologous end joining, (NHEJ).¹⁹ Its role in repair of DNA ICLs has been extensively studied.^{11–19} DNA ICL repair is a complex process and can occur in both replicating and non-replicating DNA.^{33–36} It involves a number of steps which include damage recognition and incision, translesion DNA synthesis, homologous recombination, and NER.^{45–51}

Multiple lines of evidence have demonstrated an involvement of α IISp in the repair of DNA ICLs: (1) Purified bovine brain α IISp binds directly to a DNA substrate containing 4,5',8-trimethylpsoralen (TMP) ICLs as does α IISp from HeLa cell nuclei.¹² Based on the crystal structure of α IISp, we have proposed that α IISp could interact with the DNA in its minor groove via hydrogen bonding between polar residues present on the surface of α IISp's α -helical repeat units and the N₃ of purines and O₂ of pyrimidines in DNA.¹² After ICL damage, the minor groove would open up due to formation of an ICL. This would enhance the ability of α IISp to bind to DNA after ICL damage.¹² (2) α IISp interacts with XPF, an endonuclease involved in ICL repair and production of incisions at the site of an ICL.^{14,45,48,52} After

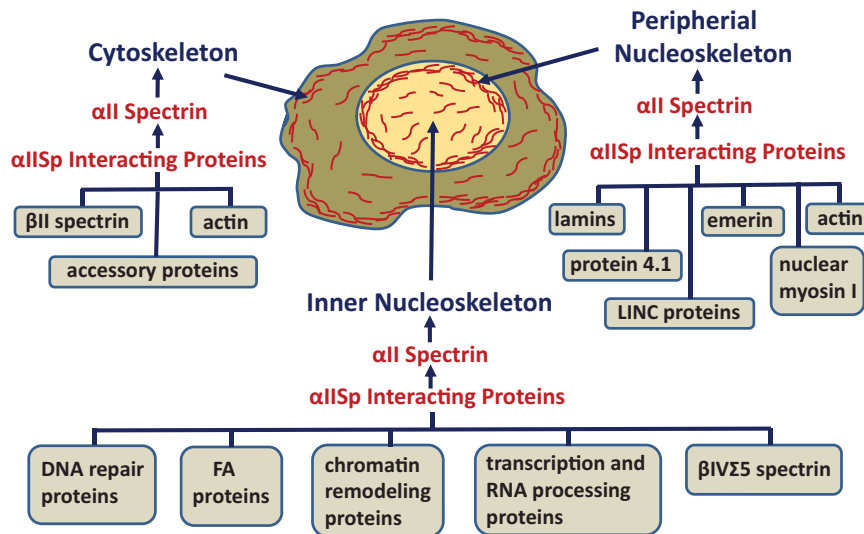


Figure 1. Overview of the localization of α IISp in normal human cells. α IISp is present in the cytoplasm where it is an important component of the cytoskeleton and interacts with a number of proteins including β II spectrin, actin and a number of accessory proteins.^{23–25,33–39} In the nucleus, it is present in the peripheral nucleoskeleton, where it interacts with lamins, emerin, actin, protein 4.1, nuclear myosin and LINC complex proteins.^{7,19–21} α IISp is also present in the inner nucleoskeleton where it interacts with DNA repair proteins, FA proteins, chromatin remodeling proteins, transcription and RNA processing proteins, and β IVS5 spectrin.¹⁹

ICL damage, α IISp co-localizes with XPF in nuclear foci (Figure 2(a)).^{13,16,17} Since α IISp binds directly to DNA containing ICLs, this indicates that these foci represent co-localization of these proteins at sites of ICLs. (3) The time course for formation of α IISp and XPF foci in the nucleus is the same, indicating that they are involved in the same steps in ICL repair (Figure 3).¹³ (4) α IISp is needed for the recruitment of XPF to sites of DNA ICLs and for production of incisions produced by XPF at these sites.^{15,17} Knockdown of α IISp by siRNA leads to a loss in localization of XPF to nuclear foci after damage with a DNA ICL agent.^{15,17} (5) Incisions produced by XPF at a site-specific TMP ICL on a DNA substrate are inhibited by a monoclonal antibody against α IISp from normal human lymphoblastoid cells which specifically recognizes and binds to α IISp.¹² Purified α IISp enhances incisions produced by XPF at sites of ICLs on this substrate.¹² These studies have led us to propose that α IISp acts as a scaffold to aid in the recruitment of XPF to sites of ICLs; in its absence, XPF is not recruited and there is loss of incisions at these sites.^{12,18} (6) α IISp is critical for chromosomal stability and cell survival after DNA ICL damage.^{15,18} Knock-down of α IISp by siRNA leads to decreased chromosomal stability and decreased cell survival after treatment with a DNA ICL agent.¹⁵ Thus, α IISp plays an important role in DNA ICL repair and its interaction with specific DNA repair proteins is critical for this process.

α IISp interacts with FA proteins; Its association with FANCA

FA is an inherited bone marrow failure disorder. One of the identifying hallmarks of this disorder is a deficiency in

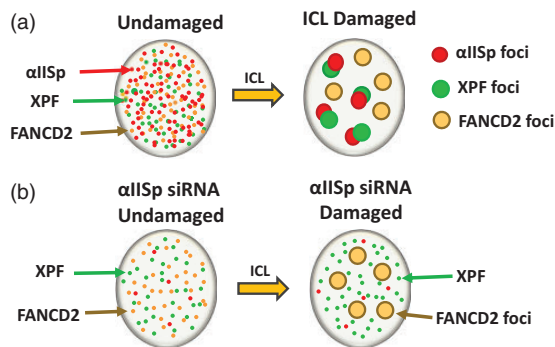


Figure 2. Diagrammatic representation of the formation of α IISp, XPF, and FANCD2 foci in the nuclei of normal human cells after damage with a DNA ICL agent. (a) In undamaged cells, α IISp, XPF, FANCD2 are localized diffusely in the nucleus; 16 h after damage with either 8-MOP plus UVA light or mitomycin C (MMC), α IISp and XPF are co-localized in nuclear foci at sites of ICLs.¹³ FANCD2 has also formed nuclear foci; however, these foci do not colocalize with α IISp foci and it is inferred from this that they do not co-localize with XPF foci.⁶⁴ Since, after DNA ICL damage, FANCD2 foci form before those of α IISp or XPF foci (at 2 h vs. 8 h) and follow a different time course for formation, we have proposed that FANCD2 acts upstream of α IISp and XPF.⁶⁴ Thus, at the same point in time after ICL damage, FANCD2 do not localize on the same sites on DNA as α IISp and XPF, hence the different localization of FANCD2 foci compared to those of α IISp and XPF. (b) In normal human cells in which α IISp has been knocked-down by siRNA, XPF and FANCD2 are localized diffusely in the nucleus; however, levels of α IISp are only ~30% of those found in non-treated cells; 16 h after ICL damage, FANCD2 is localized in nuclear foci; however, XPF does not form nuclear foci.^{13,64} Thus, α IISp is needed for formation of XPF nuclear foci and localization of XPF to sites of DNA ICLs, but it is not needed for the localization of FANCD2 to sites of damage.⁶⁴

ability to repair DNA ICLs.^{46,47,51–60} Clinically, it is characterized by bone marrow failure, developmental abnormalities, chromosomal instability, and a high predisposition to development of cancer.^{54–56,59–63} We have shown that there is a deficiency in α IISp in cells from a number of FA complementation groups.^{11,15,16} This deficiency has been shown to correlate with a defect in DNA ICL repair, as demonstrated by decreased unscheduled DNA synthesis (UDS) and decreased cell survival after damage.¹⁴ FA thus serves as an excellent model for elucidation of the effects of loss of α IISp on cellular processes in human cells.

A core complex of FA proteins is involved in the preincision steps in the ICL repair process.^{46,47,57,60,63} This complex is critical for the monoubiquitination of the FA proteins, FANCD2, and FANCI.^{46,47,57,60,63} One of the proteins in this core complex is FANCA. In addition to its role as a component of the core complex, we have shown that FANCA also interacts with α IISp and XPF at the site of damage.^{11–13} It co-localizes with both α IISp and XPF in nuclear foci in cells at sites of ICLs created by psoralen plus UVA light (Figure 4(a)) and co-immunoprecipitates with both α IISp and XPF.¹³ It also binds to a synthesized DNA substrate containing a psoralen interstrand cross-link.¹² The time course for formation of FANCA nuclear foci is the same as that for both α IISp and XPF (Figure 3).^{13,64} These three proteins co-localize in nuclear foci from 10 to 22 h after ICL damage after which time they are no longer observed (Figure 3).¹³ These studies thus demonstrate that α IISp associates with both XPF and FANCA at sites of DNA ICLs, indicating their involvement in the ICL repair process. The exact role of FANCA in this step is not yet clear.

α IISp and FANCD2 dissociate from each other after DNA ICL damage

α IISp associates with another FA protein, FANCD2, in the nucleus of normal human cells before DNA ICL damage.⁶⁴ However, after FANCD2 is monoubiquitinated in the initial steps of DNA ICL repair, FANCD2 and α IISp dissociate from each other.⁶⁴ We have shown that following ICL damage, FANCD2 localizes to nuclear foci which form before those of α IISp. α IISp and FANCD2 do not co-localize in the same foci (Figure 2(a)).⁶⁴ Formation of FANCD2 foci follows a different time course than those of α IISp, XPF, and FANCA (Figure 3).⁶⁴ FANCD2 localizes to foci that appear 2 h after damage with the DNA ICL agent, 8-MOP plus UVA light, plateau at 16 h and are still present at 72 h after damage.⁶⁴ In contrast, α IISp, XPF, and FANCA foci are first observed at 10 h after damage, peak at 16 h and are no longer observed by 24 h after ICL damage (Figure 3). This indicates that FANCD2 is recruited to sites of damage before α IISp, XPF, and FANCA and acts upstream of all three proteins.⁶⁴ Results similar to these have been obtained using *Xenopus* extracts.⁴⁸ These studies showed that FANCD2 is recruited to sites of damage before XPF.⁴⁸ Collectively, these studies indicate that following DNA ICL damage and monoubiquitination of FANCD2, α IISp, XPF, and FANCA co-localize to sites of damage and act downstream of FANCD2 and that α IISp and FANCA play

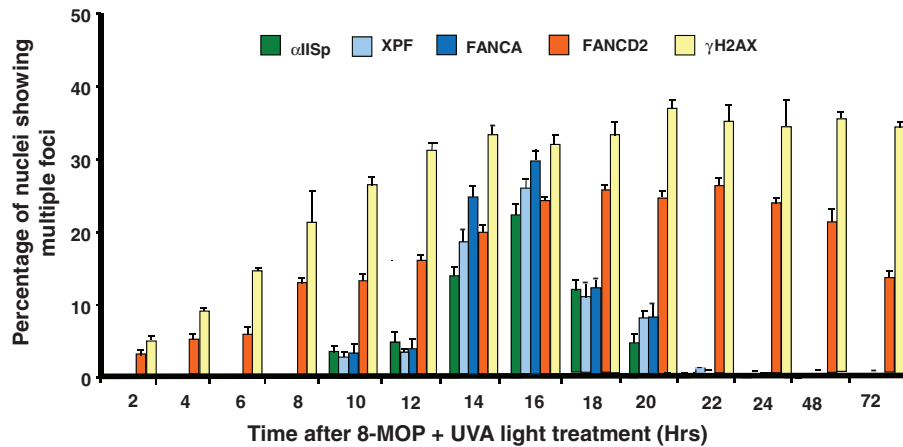


Figure 3. Compiled time course for appearance of α IISp, XPF, FANCA, FANCD2, and γ -H2AX nuclear foci after damage with a DNA ICL agent. After treatment of normal human cells with a DNA ICL agent, 8-MOP plus UVA light, and, at the indicated time after DNA damage, the percentage of nuclei showing multiple α IISp, XPF, FANCA, FANCD2, and γ -H2AX foci was determined at time points from 0 to 72 h after treatment. At each time point, nuclear foci for 100 nuclei were counted. Nuclei were counted as positive if they contained four or more foci. Error bars represent SEM. (Modified from Zhang *et al.*⁶⁴ with permission from Wiley Periodicals, Inc. Notations of XPF and FANCA foci from Sridharan *et al.*¹³).

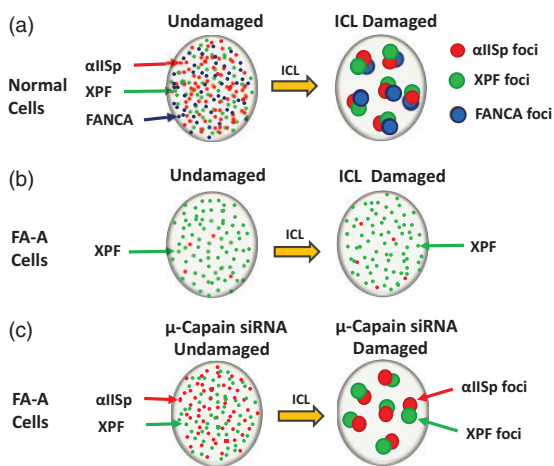


Figure 4. Diagrammatic representation of the formation of α IISp, XPF, and FANCA foci in the nuclei of normal human cells and FA-A cells after damage with a DNA ICL agent. (a) In undamaged cells, α IISp, XPF, and FANCA are localized diffusely in the nucleus; 16 h after damage with either 8-MOP plus UVA light or MMC, α IISp, XPF, and FANCA co-localize with each other in nuclear foci at sites of ICLs.¹³ (b) In FA-A cells, in which levels of α IISp are ~30% of normal levels, XPF is localized diffusely in the nucleus. FANCA is not discernable and there are very low levels of α IISp; 16 h after ICL damage, XPF does not form nuclear foci and there are few α IISp foci.^{13,64} This indicates that α IISp is needed for formation of XPF nuclear foci and localization of XPF to sites of DNA ICLs.⁶⁴ (c) In FA-A cells, after treatment with μ -calpain siRNA, levels of α IISp are restored to normal and both α IISp and XPF are localized diffusely in the nucleus.¹⁶ Sixteen hours after damage with an ICL agent, MMC, both α IISp and XPF foci form and these foci co-localize at sites of damage.¹⁶ This indicates that α IISp is needed for the formation of XPF foci at sites of DNA ICLs.

a role with XPF in the incision step in ICL repair. They also indicate that FANCD2 is involved in an earlier step in this process as well as in subsequent steps in the ICL repair process, such as homologous recombination, as has been proposed.⁴¹

Our studies in normal human cells have also shown that α IISp is not needed for the monoubiquitination of FANCD2.⁶⁴ Knock-down of α IISp has no effect on the localization of FANCD2 to nuclear foci or chromatin.⁶⁴ This indicates that α IISp is not needed for the functioning of

monoubiquitinated FANCD2 (FANCD2-Ub), which further indicates that it acts downstream of FANCD2-Ub. Studies using *Xenopus* extracts have similarly shown that XPF is not required for the monoubiquitination of FANCD2.⁴⁸ Thus, two proteins shown to play a role in DNA ICL repair, α IISp, and XPF are targeted to the same site after ICL damage and this site is different from that of FANCD2-Ub and is downstream of that of FANCD2.

FANCG binds to both α IISp and XPF-ERCC1

We have demonstrated, using yeast two-hybrid analysis as well as co-immunoprecipitation, that FANCG has strong binding affinity for α IISp.³² It has a consensus sequence that binds to the Src-homology 3 (SH3) domain of α IISp.³² SH3 domains are modular domains that are involved in protein-protein interactions and assembly of protein networks involved in intracellular signaling, protein synthesis, and cellular organization.^{29–31} Three major classes of protein ligands bind to SH3 domains: class I, class II, and class 1@.^{31,65–69} The SH3 domain of α IISp preferentially binds to class 1@ ligands.⁶⁹ A number of FA proteins have motifs with consensus sequences that can bind to SH3 domains.³² These motifs represent another important class of motifs in FA proteins that could interact with cellular proteins containing SH3 domains, such as those involved in signal transduction and intracellular signaling.³² FANCG contains a class 1@ consensus sequence.³² Of particular interest, we have shown that FANCG specifically binds to the SH3 domain of α IISp via this consensus sequence.³² FANCG also has binding affinity to XPF-ERCC1.³² It contains seven tetratricopeptide repeat (TPR) motifs, which are motifs involved in protein-protein interactions.^{70–73} We have shown that TPRs 1, 2, 3, and 6 are important for binding of full length FANCG with the central domain of ERCC1 (residues 120–220).⁷⁴ ERCC1 binds to XPF via its C-terminal domain (residues 220–297), which is different from the nuclease domain of XPF that is involved in production of incisions at sites of damage.^{75–77} Thus, we

propose that FANCG, through its binding to both α IISp and XPF/ERCC1, acts as a link between α IISp and XPF/ERCC1 at sites of damage.

Model for α IISp's role in DNA ICL repair and its mechanism of action

Based on evidence described above that α IISp is critical for repair of DNA ICLs, we have proposed a model for the role of α IISp in DNA ICL repair (Figure 5). According to this model, when DNA replication is blocked at the site of an ICL, a FA core complex comprising FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and associated

proteins, in conjunction with FANCM, is recruited to the damaged DNA.^{46,47,60,78–80} The FA core complex, through its E2-ubiquitin conjugating activity and E3-ubiquitin ligase activity, leads to the monoubiquitination of FANCD2 (FANCD2-Ub) and FANCI (FANCI-Ub) to form a heterodimer known as ID2.^{46,47,79,81} The ID2 heterodimer localizes to the damaged DNA.^{46,47,79,81} α IISp is recruited and binds to DNA at the site of the ICL downstream of and at a different site than ID2.^{12,64} It is not known whether FANCD2-Ub or ID2 is involved in recruitment of α IISp to sites of ICLs. FANCG binds to α IISp and recruits XPF-ERCC1 which binds to FANCG.^{32,74} Thus, α IISp, via its binding to FANCG, aids in the recruitment of XPF-ERCC1

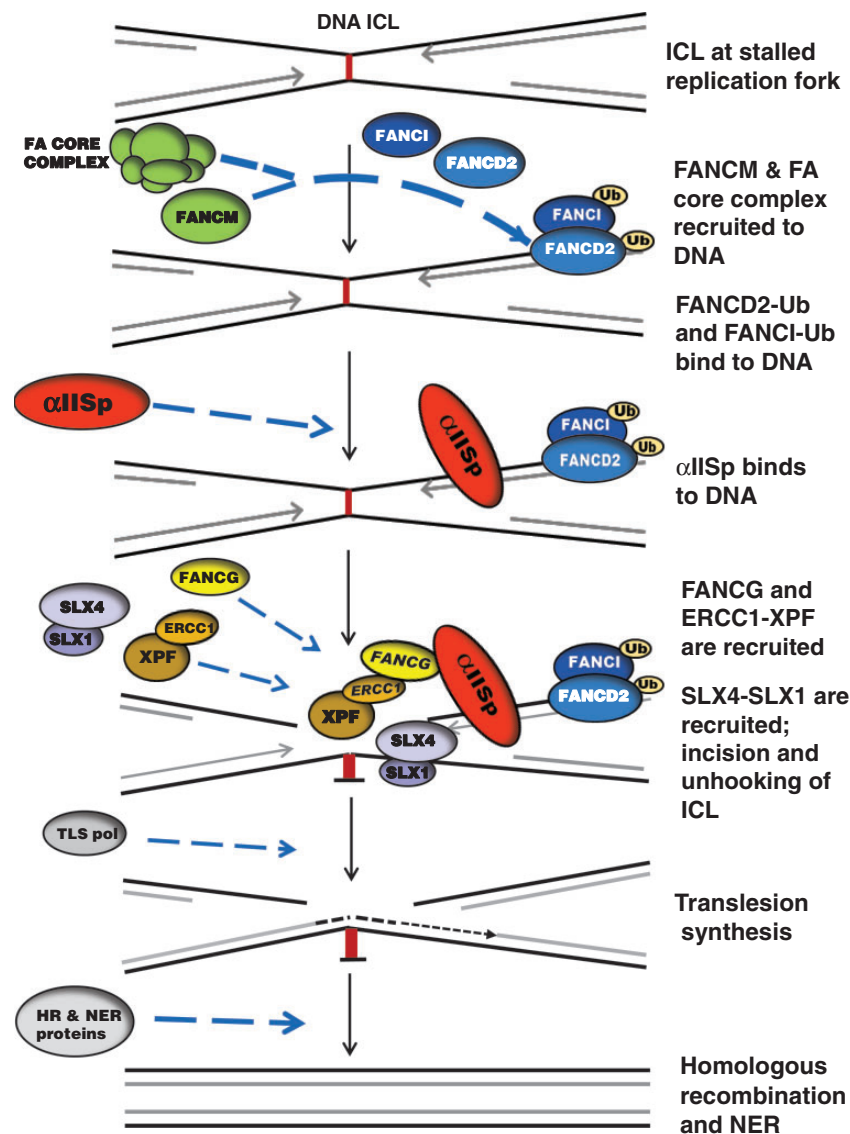


Figure 5. A model for α IISp's role in DNA ICL repair and a mechanism of action proposed by our laboratory. When DNA replication is stalled at the site of an ICL, the FA core complex, in conjunction with FANCM, is recruited to the DNA.^{46,60,78–80} The core complex monoubiquitinates FANCD2 and FANCI (FANCD2-Ub and FANCI-Ub), which form a heterodimer (ID2) that binds to the damaged DNA.^{46,47,79,81} α IISp is recruited to the DNA at the site of the ICL and binds to the DNA downstream from the ID2 complex and at a different site.^{12,64} FANCG binds to the SH3 domain of α IISp via a consensus sequence that recognizes SH3 domains.³² FANCG in turn recruits XPF-ERCC1 to the site of damage.⁷⁴ FANCG binds to the central domain of ERCC1; the TPR motifs of FANCG are involved in this interaction.⁷⁴ Thus, α IISp, via its binding to FANCG, aids in the recruitment of XPF-ERCC1 to the site of an ICL. XPF-ERCC1 is then involved in the incision of DNA at this site in conjunction with other nucleases such as SLX4-SLX1.^{46–49,80} This leads to unhooking of the ICL. Translesion DNA synthesis then takes place by a translesion DNA polymerase (TLS pol).^{46,47,60,61,80} In subsequent steps, the DNA duplex is repaired by homologous recombination (HR) via a series of downstream proteins and the adducted base is removed by nucleotide excision repair (NER).^{46,47,57,80} Additional FA proteins and other repair proteins involved in these steps are not shown in this model since the emphasis has been put on where α IISp fits into the ICL repair process.

to the site of an ICL. XPF-ERCC1 is then involved in the incision step of ICL repair in conjunction with the nuclease/protein complex, SLX4-SLX1, leading to unhooking of the ICL.^{45–49,80} Other nucleases may also be involved in this step.^{46,47,49,80} The importance of α IISp in recruitment of XPF-ERCC1 is demonstrated by the loss of localization of XPF to sites of ICLs when α IISp is knocked down (Figure 2 (b)).¹⁵ Translesion DNA synthesis then takes place by a multi-subunit translesion polymerase.^{46,47,60,61,80} In the later stages of ICL repair, the broken DNA duplex is repaired by homologous recombination by the cooperative functioning of a series of downstream proteins and the adducted DNA base is removed.^{46,47,57,80} The additional FA proteins and other repair proteins involved in these later steps, as well as in earlier steps in the repair process, are described in a number of reviews^{43,46,58,60,80} and are not shown in this model since the emphasis has been put on where α IISp fits into the ICL repair process.

Our combined studies thus propose a mechanism of action for α IISp in its role in DNA repair acting as a scaffold to recruit proteins to sites of damage: α IISp directly binds to DNA at the site of a DNA ICL; it directly binds, via its SH3 domain, to FANCG, and FANCG in turn directly binds to ERCC1. XPF, through its binding, is thus recruited, via α IISp, to the site of damage where it can incise the DNA.

Regulation of α IISp stability by FA proteins

α IISp is an essential protein in cells and its depletion can lead to cell death.^{15,37,38,82,83} Therefore, maintaining its stability in cells is critical. We have evidence that FA proteins aid in this process and help maintain cellular levels of α IISp.¹⁶ We have proposed that this is accomplished by regulation of cleavage of α IISp by the protease, μ -calpain.¹⁶ In cells, μ -calpain cleaves α IISp into distinct cleavage products and this process is important for a number of functions mediated by α IISp.^{84–87}

Increased activity of μ -calpain can lead to enhanced cleavage of α IISp which has deleterious effects on a number of cellular processes.^{84–87} We have shown that in cells from a number of FA complementation groups, FA-A, FA-C, FA-D2, FA-F, and FA-G, there is a 2.5–3.5 fold increase in μ -calpain activity compared to normal cells.¹⁶ This correlates with increased cleavage of α IISp in these cells.¹⁶ We have proposed that FA proteins play a critical role in maintaining α IISp stability in cells and preventing its cleavage by μ -calpain.^{16,18} We have developed a model to explain the role of FA proteins in this process.

Model for the role of FA proteins in maintaining α IISp stability

There are several possible mechanisms by which FA proteins could reduce cleavage of α IISp by μ -calpain. One mechanism we propose is that in normal cells, an FA protein such as FANCG binds to the SH3 domain of α IISp and this in turn leads to decreased ability of μ -calpain to cleave α IISp (Figure 6).¹⁶ In support of this, a number of FA proteins contain motifs with a consensus sequence that recognizes SH3 domains.³² We have shown that FANCG has a consensus sequence that specifically recognizes and binds to the SH3 domain of α IISp.³² An equilibrium would exist among the FA protein (i.e. FANCG), low-molecular weight phosphotyrosine phosphatase (LMW-PTP), and the kinase, c-Src for binding to the SH3 domain.¹⁶ When c-Src binds to the SH3 domain, it phosphorylates Tyr¹¹⁷⁶ at the μ -calpain cleavage site and prevents cleavage of α IISp by μ -calpain.^{86,88} When LMW-PTP binds to the SH3 domain, Tyr¹¹⁷⁶ becomes dephosphorylated, enabling μ -calpain to cleave α IISp (Figure 6).^{86,88} Binding of the FA protein to the SH3 domain would then prevent binding of LMW-PTP to this site, and inhibit its ability to dephosphorylate Tyr¹¹⁷⁶, thus inhibiting cleavage of α IISp by μ -calpain.¹⁶ Of particular interest, at least 18 patient-derived mutations

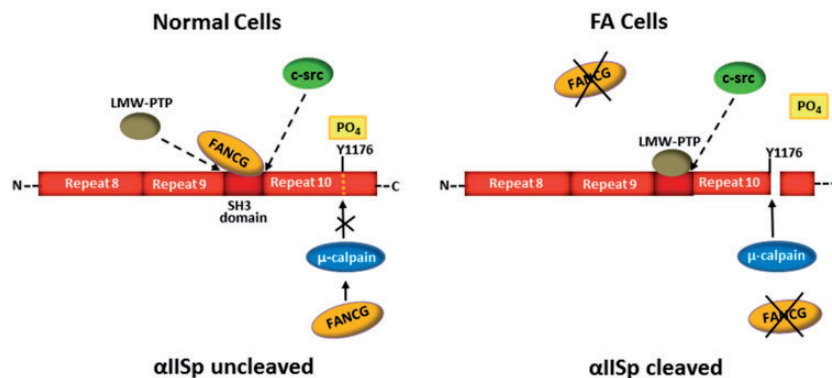


Figure 6. A model proposed for the regulation of α IISp stability by FA proteins. This model proposes that FA proteins play a critical role in maintaining the stability of α IISp in the nucleus of normal human cells by regulating its cleavage by the protease, μ -calpain. There are several possible mechanisms by which this can be accomplished; one is presented here. In this figure, a segment of α IISp, containing repeats 8–10, is shown. FANCG is used to illustrate one potential role of an FA protein in this process in normal human cells. An equilibrium exists among c-Src, LMW-PTP, and FANCG for binding to α IISp at its SH3 domain.¹⁶ Binding of c-Src to the SH3 domain leads to phosphorylation of Tyr¹¹⁷⁶ (Y1176) at the μ -calpain cleavage site in repeat 10 of α IISp, preventing cleavage of α IISp by μ -calpain.^{86,88} When LMW-PTP binds to the SH3 domain, Tyr¹¹⁷⁶ becomes dephosphorylated, enabling μ -calpain to cleave α IISp.^{86,88} Binding of FANCG to the SH3 domain blocks binding of LMW-PTP to the SH3 domain and prevents cleavage of α IISp by μ -calpain.¹⁶ An FA protein may also inhibit α IISp cleavage by binding to μ -calpain and inhibiting its ability to cleave α IISp.¹⁶ In FA cells (for example, FA-G cells), the absence of FANCG would lead to a greater probability that LMW-PTP would bind to the SH3 domain of α IISp, leading to dephosphorylation of Tyr¹¹⁷⁶ and cleavage of α IISp by μ -calpain. FANCG would also not be present to additionally bind to μ -calpain and inhibit its cleavage of α IISp. In other FA complementation groups, similar events could occur. (Modified from Zhang *et al.*¹⁶ with permission from the American Chemical Society).

have been identified in *FANCG* which would result in the *FANCG* protein either missing or having a defect in the motif that binds to the SH3 domain of α IISp.³² This would increase binding of LMW-PTP to the SH3 domain of α IISp leading to increased cleavage by μ -calpain. Thus, we propose that maintenance of normal levels of FA proteins in cells is critical for maintenance of normal levels of uncleaved α IISp.

Another mechanism by which FA proteins may inhibit α IISp cleavage by μ -calpain is by binding to μ -calpain and inhibiting its activity. In support of this view are our yeast two-hybrid data showing that *FANCA* and *FANCG* bind directly to μ -calpain.¹⁶ A third mechanism by which FA proteins could potentially regulate α IISp cleavage would be by modulating binding of calmodulin to α IISp. When calmodulin binds to a site on α IISp adjacent to μ -calpain's cleavage site, this leads to enhanced cleavage of α IISp by μ -calpain.⁸⁹⁻⁹¹ An FA protein could potentially bind to calmodulin inhibiting its binding to α IISp and thus could decrease μ -calpain activity and α IISp cleavage. Thus, there are a number of ways in which FA proteins could regulate α IISp stability and reduce its cleavage by μ -calpain. This suggests another potential role for FA proteins in the cell, that of maintenance of α IISp stability.

Consequences of loss of FA proteins

In FA cells, deficiency in a specific FA protein correlates with reduced levels of α IISp and reduced localization of a repair protein such as XPF to sites of damage (Figure 4 (b)).¹¹ When levels of these FA proteins are restored by transfection of FA cells with the appropriate FA c-DNA, α IISp levels in these corrected cells (FA-A+, FA-C+, FA-G+) are similar to normal.^{11,16} Thus, there is a correlation between α IISp level in cells and the presence of a specific FA protein. These studies further strengthen the view that FA proteins are important in maintaining α IISp stability.¹⁶⁻¹⁸ Different FA proteins could have different roles in modulating α IISp stability and reducing its cleavage by μ -calpain. This could occur through their interactions, either directly or indirectly, with α IISp, μ -calpain or possibly calmodulin.

Knock-down of μ -calpain in FA cells restores α IISp levels to normal

We have proposed that if the increased activity of μ -calpain in FA cells is reduced, then levels of α IISp could be returned to normal and DNA repair should be enhanced.¹⁶ In support of this view, we have shown that knock-down of μ -calpain by siRNA in FA-A cells leads to an increase in α IISp levels to those similar in normal cells and to correction of chromosomal instability, to formation of XPF foci at sites of DNA damage (Figure 4(c)), to ability to repair DNA ICLs and to increased cell survival after damage.¹⁶ Thus, by knocking down μ -calpain and restoring levels of α IISp to normal in FA-A cells, we were able to restore a number of the phenotypic characteristics of these FA-A cells to normal.¹⁶ Levels of the *FANCA* protein, however, were not increased and there was still a deficiency in this protein in FA-A cells.¹⁶ We have proposed that when restoration of

α IISp levels in FA cells is achieved by an alternate means (i.e. knocking-down μ -calpain), these cells are then able to carry out functions they could otherwise not accomplish such as DNA repair and maintenance of chromosome stability and telomere function after ICL damage.^{16,18} Thus, in these FA cells, the presence of a specific FA protein becomes less critical, since an endpoint of its function (i.e. maintenance of α IISp stability) has been achieved by another means.

α IISp prevents telomere dysfunction after DNA ICL damage

α IISp interacts with telomeres

Telomeres are specialized nucleoprotein structures at the ends of chromosomes which are critical for preserving genomic integrity.⁹²⁻⁹⁴ Damage to telomeric DNA, if left unrepaired, can lead to telomere dysfunction and chromosomal instability.⁹²⁻⁹⁴ A multiprotein complex, shelterin, binds to telomeres and helps protect them and prevent telomere dysfunction.^{92,94-96} Repair of damage to telomeric DNA is thus essential for insuring the integrity of telomeric DNA and maintenance of chromosome stability. We have shown that α IISp is important in this repair process.¹⁷ It is recruited to telomeres after DNA ICL damage where it co-localizes with two proteins in the shelterin complex, TRF1, and TRF2.¹⁷ α IISp is also needed for the recruitment of XPF to telomeres after ICL damage and it co-localizes with XPF at these sites of damage.¹⁷ Knock-down of α IISp by siRNA results in loss of localization of XPF to telomeres after damage.¹⁷ These studies thus demonstrate that α IISp plays a role in the recruitment of XPF to sites of DNA damage in telomeres and in repair of DNA ICLs at these sites.

Telomeres replicate in S phase.^{92-94,97} Since production of ICLs in DNA can lead to stalled replication forks,⁴¹⁻⁴⁴ it is thus essential that, at the time of replication, damage occurring in telomeric DNA is repaired so that DNA replication can be re-initiated. Of particular interest, α IISp specifically localizes to telomeres in S phase indicating its importance in the DNA repair response at the site of a stalled replication fork.¹⁷

α IISp is needed for telomere function after DNA ICL damage

α IISp is also critical for maintenance of telomere function after ICL damage.¹⁷ We have demonstrated that knock-down of α IISp in normal human cells leads to telomere dysfunction.¹⁷ This can be assessed by examination for the presence of telomere dysfunction-induced foci (TIF) after ICL damage, which can be determined by quantitation of γ -H2AX foci.¹⁷ These foci represent sites of DNA double-strand breaks (DSBs) that arise when replication forks are stalled at sites of ICLs and fail to restart.⁹⁸ They are used as an indicator of dysfunctional telomeres.⁹⁸ In normal human cells, loss of α IISp by knock-down leads to an increased number of TIF positive cells after ICL damage.¹⁷ Another indicator of telomere dysfunction after ICL damage is an increase in chromosomal aberrations. Knock-down of α IISp

in normal cells leads to an increase in chromosomal aberrations, particularly sister chromatid end-to-end fusions.¹⁷ A third indicator of telomere dysfunction is loss of telomeres. This is proposed to occur when telomeres are stalled at replication forks and the replication forks collapse.^{92,93} DSBs then form in DNA, leading to breakage of telomeres and their loss. We have demonstrated that, after knock-down of α IISp in normal human cells and exposure of these cells to an ICL agent, there is a catastrophic loss of telomeres (Figure 7(a) and (b)).¹⁷ We hypothesize that reduced levels of α IISp in these cells prevent effective repair of DNA ICLs in telomeric DNA during S phase and that this results in stalling of the replication fork,

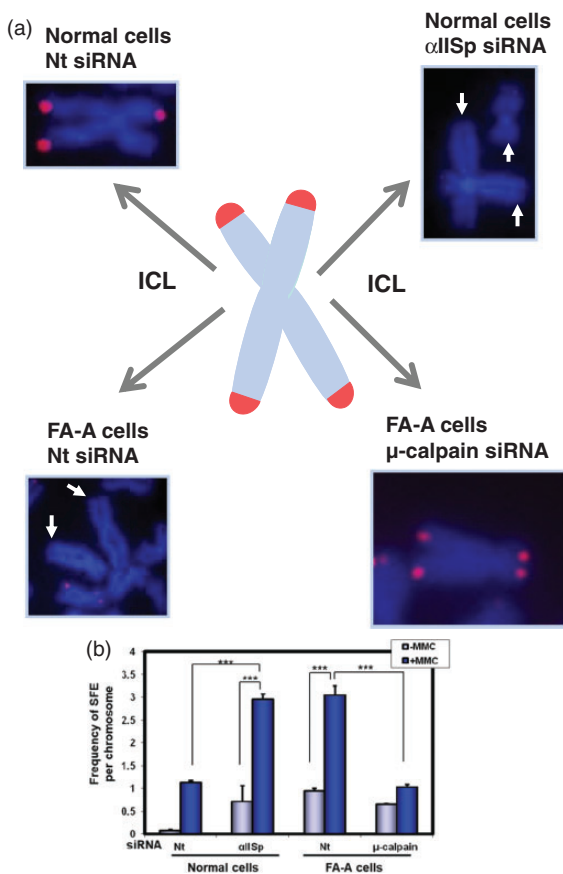


Figure 7. A deficiency in α IISp results in loss of telomeres after DNA ICL damage. (a) In normal human and FA-A cells, after treatment with MMC, metaphase chromosomes were stained with DAPI (blue).¹⁷ Telomeric DNA was identified using FISH with a Cy3-labeled telomere specific PNA probe (red).¹⁷ In normal cells transfected with α IISp siRNA, so as to knock-down α IISp, and in FA-A cells transfected with non-target (Nt) siRNA, levels of α IISp, in both groups of cells, are ~30% of those in normal cells. After treatment of these cells with MMC, there is an increased loss of telomeres (or the presence of signal free ends (SFEs)) in telomeres.¹⁷ Arrows point to signal free ends (SFEs) in telomeres. After FA-A cells are transfected with μ -calpain siRNA, so as to increase levels of α IISp to those present in normal cells, and subsequently treated with MMC, the frequency of SFEs in chromosomes was reduced to levels similar to those in normal cells transfected with Nt siRNA. (Chromosome inserts are reproduced from Zhang *et al.*¹⁷ with permission of Oxford University Press) (b) Quantitation of the frequency of SFEs per chromosome for normal and FA-A cells was carried out. Means of five independent experiments are shown in which 4600 chromosomes per experiment were scored for telomeric signals on each of the chromatids. These results indicate that α IISp plays a critical role in maintenance of telomere function after DNA ICL damage.¹⁷ Error bars are S.E.M. *** $P < 0.0001$. (Reproduced from Zhang *et al.*¹⁷ with permission from Oxford University Press).

formation of DSBs in telomeric DNA, and a significant loss of telomeres.¹⁷ Thus, these studies collectively demonstrate that α IISp plays a critical role in maintenance of telomere function after DNA ICL damage and provides evidence for another important role for α IISp in the nucleus.

α IISp loss in FA cells correlates with telomere dysfunction after ICL damage

Cells from patients with FA serve as an excellent model for the effects of loss of α IISp on telomere function. We have shown that there is a significant increase in telomere dysfunction in FA-A cells after DNA ICL damage.¹⁷ Two important findings support this view: First there is an increase in the number of TIF positive cells in FA cells (FA-A) after ICL damage as well as a significant loss of telomeres (Figure 7(a) and (b)).¹⁷ Importantly, when levels of α IISp are returned to normal by knocking down μ -calpain, the number of TIF positive cells is reduced to normal levels as is the number of chromosomes with a loss of telomeres (Figure 7(a) and (b)).¹⁷ Second, in FA-A cells, co-localization of XPF with telomeres (TRF1) is not observed after ICL damage.¹⁷ However, after restoration of α IISp levels to normal by knocking down μ -calpain, normal levels of XPF localize to telomeres.¹⁷ These studies using FA-A cells again importantly demonstrate that α IISp plays a critical role in telomere maintenance after ICL damage.

α IISp is essential for chromosome stability

Interaction of α IISp with FA and DNA repair proteins is essential for maintenance of its stability and the role it plays in repair of ICLs during DNA replication in both genomic and telomeric DNA.^{13,16,21} These processes are critical for maintenance of genomic stability. We have shown that loss of α IISp from cells leads to increased chromosomal aberrations after DNA ICL damage.^{15,17} This is clearly observed in normal human cells in which α IISp has been knocked-down by siRNA.^{15,17} These aberrations include sister chromatid end-to-end fusions, chromatid breaks, chromosome exchanges, and radials.^{15,17} Similar chromosomal aberrations have been observed in FA cells after ICL damage.⁵⁴⁻⁵⁶ Since levels of α IISp in FA cells are 35-40% of normal and are similar to levels in normal cells in which α IISp has been knocked down, this indicates that a reduction in levels of α IISp in cells can have a significant effect on chromosome stability after DNA ICL damage. These changes correlate with loss of repair of DNA ICLs that occurs in both genomic and telomeric DNA.^{15,17} Of importance, the chromosomal aberrations observed in FA-A cells after ICL damage are corrected when μ -calpain is knocked down resulting in return of α IISp levels in the nucleus to normal.¹⁶ Studies have also shown that translational repression of *SPTAN1*, the gene which codes for α II spectrin, by miR-128-3p results in chromosome instability, acceleration of cell cycle arrest, and a reduced DNA damage response after MMC treatment.⁹⁹ These studies, collectively, further accentuate the critical role α IISp plays in maintenance of chromosome stability after ICL damage.

Nuclear beta spectrins

In the cytoplasm, α II-spectrin interacts with β -spectrins to form a tetramer which, as mentioned above, is important in maintaining cell architecture and plasma membrane stability as well as in a number of other cellular functions.^{23–25,33–39} This type of interaction has not as yet been observed in the nucleus. However, several β -spectrins have been described in the nucleus. Two isoforms of β IV-spectrin have been identified in the nucleus of a variety of mammalian cell types: a truncated, 77 kDa major isoform, Sp β IV Σ 5, and a full-length minor isoform, Sp β IV Σ 1.¹⁰⁰ An antibody developed by Tse *et al.*, which recognizes both isoforms of β IV spectrin, shows that, on Western blot analysis, Sp β IV Σ 5 is the major band observed and Sp β IV Σ 1 is present in very faint bands.⁹⁸ Indirect immunofluorescence studies show that Sp β IV Σ 5 co-localizes in the nuclear matrix with promyelocytic leukemia (PML) bodies.¹⁰⁰ The function of Sp β IV Σ 5 in the nucleus is not yet clear; however, evidence suggests that it contributes to the formation of PML bodies.¹⁰⁰ Sp β IV Σ 1 is diffusely distributed throughout the nuclear matrix.¹⁰⁰ It has a conserved actin/4.1-binding domain, which is hypothesized to bind to actin and protein 4.1 in the nucleus.¹⁰⁰ Sp β IV Σ 1 is further hypothesized to contribute to the formation of the nuclear skeleton.¹⁰⁰ Our studies, using normal human lymphoblastoid cells and the antibody developed by Tse *et al.*¹⁰⁰ against Sp β IV, have shown that Sp β IV localizes in major foci, presumed to be PML bodies, and in a fine reticular pattern throughout the nuclear matrix (unpublished data), in agreement with the findings of Tse *et al.*¹⁰⁰ We have additionally shown that α II-spectrin co-immunoprecipitates with Sp β IV Σ 5 and with PML protein from nuclear extracts.¹⁹ These studies, combined with those of Tse *et al.*,¹⁰⁰ suggest that both α II spectrin and Sp β IV Σ 5 play a role in PML body formation/function. Since Sp β IV Σ 1 is a full-length protein with actin/4.1-binding sites and is postulated to be a component of the nuclear matrix,¹⁰⁰ we hypothesize that the interaction we have demonstrated between α II spectrin, actin and protein 4.1 in the nucleus¹⁹ could be via association of α II spectrin with Sp β IV Σ 1. This suggests exciting new possibilities regarding the interaction α II spectrin with β -spectrins in the nucleus which need to be further explored.

Another β -spectrin, termed β 2-spectrin (β 2SP, gene *Sptbn1*),^{101–103} which is a TGF- β /Smad3/4 adaptor protein and transcriptional cofactor that can regulate TGF- β signaling, has been shown to undergo nuclear translocation and have nuclear functions in mouse embryonic fibroblasts (MEFs).^{101–104} Studies indicate a role for β 2SP in genomic stability and DNA repair.^{101–104} *Sptbn1*-homozygous-null mouse embryonic fibroblasts, following exposure to different genotoxic agents such as ionizing radiation, mitomycin C, hydroxyurea, and ethanol-derived acetaldehyde, exhibit a significant increase in chromosomal aberrations and reduced cell survival compared to wild type MEFs.^{101–104} Studies suggest that these β 2SP depleted cells have a defect in DNA double-strand break repair by homologous recombination and that this repair defect is specific for S-phase.^{101–104} Thus, β 2SP may have an important role in the DNA damage response just as has been demonstrated

for α IISp. Since β II-spectrin in the cytoplasm has binding sites for actin and for protein 4.1 and also binds to α II-spectrin,^{23–25} it could be hypothesized that, in the nucleus, α II-spectrin similarly binds to β II-spectrin and that it is through this interaction that it achieves a functional association with actin and protein 4.1. There is as yet, however, no reported evidence for an interaction between α II-spectrin and β II-spectrin in the nucleus of human cells. Collectively, the studies described above point to the possibility of association of α II-spectrin with full length β -spectrins in the nucleus and that more than one form of β -spectrin may be involved. Such an association could be an important component of α II-spectrin's structural role in the nucleus.

α IISp interacts with structural proteins in the nucleoskeleton

In addition to its interaction with proteins in the nucleus involved in DNA repair and its role in the DNA repair response and in chromosome stability, α IISp also has a role in functioning of the nucleoskeleton.^{6,7,19–22} It interacts with structural proteins in the nucleoskeleton, which constitutes a network of interacting components including the lamins (types A and B), actin, emerin, protein 4.1, nesprin (spectrin-repeat domains), myosins, kinesins, titan, and β SpIV.^{7,19–21} Of these nucleoskeletal proteins, α IISp has been shown, thus far, to interact with lamin A, actin, emerin, protein 4.1, nuclear myosin 1c, and β SpIV Σ 5.^{19–21} The potential functional importance of the interaction of α IISp with these specific nucleoskeletal proteins will be discussed below.

Major proteins in the nucleoskeleton and their function

The nucleoskeleton is a structurally and functionally complex network of proteins that confers mechanical properties and functionality to the nucleus and the genome.^{6,7,20,21,105} It is composed of two major components: a peripheral component concentrated near the nuclear envelope and an internal component that extends throughout the interior of the nucleus and consists of more loosely associated proteins.^{7,21} There are functional differences between these two components of the nucleoskeleton. Numerous reviews have been written regarding the array and localization of proteins comprising the nucleoskeleton and their role in nuclear function.^{6,7,20,21} This section will briefly review those proteins which have, thus far, been shown to associate with α IISp.

Among the foremost of these nucleoskeletal proteins are the lamins. A-type and B-type lamins concentrate underneath the inner nuclear membrane where they form the nuclear lamina network.⁷ They are also distributed loosely throughout the nucleoplasm.^{3,7,106–110} They are essential components of the nuclear architecture and are critical for many aspects of normal nuclear function and maintenance of nuclear integrity.^{3,107–110} They have a broad range of functions due to their interactions with proteins that function in a variety of cellular pathways.^{3,7,20,109,110} Lamin networks make an important contribution to

mechanical support and stiffness of the nucleus, to mechano-responsive functions of the nucleus and to mechano-transduction.^{3,105,107,108,111} Lamins interact with numerous proteins in the nucleoskeleton including actin, emerin, protein 4.1, spectrin, myosin 1c, SUN2, kinesin, titin, and the nuclear mitotic apparatus.^{7,19–21} They regulate and support protein complexes involved in gene expression, DNA replication, transcription, chromatin organization, differentiation, DNA repair, epigenetic regulation, and signaling.^{3,7,107,108} Lamins thus contribute, either mechanically or non-mechanically, to a variety of cellular functions.

Actin, another protein associated with the nucleoskeleton, is present both in a monomeric or globular form (G-actin) and in a polymerized filamentous form (F-actin).^{7,112–115} Actin interacts functionally with lamin complexes, which may aid in actin filament formation.^{7,12,116,117} It also binds to emerin, protein 4.1, and α II spectrin.^{19,20} Perinuclear actin and nuclear lamins help mediate the nuclear response to mechanical stress and contribute to nuclear shape.^{20,21,118,119} Actin has many diverse roles in the nucleus which include transcription, nuclear export, chromatin remodeling, chromosome relocation, gene expression, mRNA processing, nuclear envelope assembly, and DNA repair.^{7,20,112,114,116} Thus, like the lamins, it has both structural and non-structural roles.

Emerin is a nuclear membrane protein associated with the nucleoskeleton.^{20,109,120} It localizes primarily in the inner membrane of the nuclear envelope and binds directly to lamins.^{20,109,120} Emerin helps connect the nuclear lamina to the nuclear envelope and stabilizes the lamin network, contributing to nuclear lamina structure and function.^{20,109,120–123} Purification of emerin containing complexes from HeLa cell nuclei has revealed a protein complex which includes α II-spectrin, actin, nuclear myosin, lamins A and B, and SUN2.^{20,21} It has been suggested that an important function of emerin is to interlink actin-nuclear myosin-spectrin complexes with the A- type and B- type lamins at the nuclear envelope and to help maintain the structural integrity of the nucleus.^{20,21} Emerin has roles in chromatin organization and tethering chromosomes to the nuclear envelope, promoting formation of nuclear actin filaments, nuclear assembly, gene regulation, mitosis, development, signaling, and mechano-transduction.^{20,21,109,120–122} Protein 4.1, another component of the nucleoskeleton, is enriched at the nuclear envelope.^{7,121,124} It is thought to be an important component of the cortical network, connecting actin, α II-spectrin, emerin, and A-type lamin to the nuclear envelope and in doing so has an impact upon nuclear structure and architecture.^{7,20,121,124} Thus, the nucleoskeleton is important for maintaining the shape and functional integrity of the nucleus and it is the interaction between these, as well as other nucleoskeletal proteins, which plays a critical role in these processes.

α II-spectrin's interaction with nucleoskeleton proteins

α IISp interacts with a number of these nucleoskeletal proteins. It is present throughout the nucleus in both the peripheral and internal nucleoskeleton.^{7,13,19–21,64} In the peripheral nucleoskeleton, it is observed as an intense

band just beneath the nuclear envelope (Figure 8(a))^{13,64} where it is associated with other nucleoskeletal proteins (Figure 8(b)).^{7,20,21} It has been proposed that spectrin forms a cortical network with lamin and actin filaments that is anchored by emerin and protein 4.1 to the nuclear envelope and is important for its support.^{7,20,21} Its co-immunoprecipitation with lamin A, actin, emerin, protein 4.1 and β SpIV Σ 5,¹⁹ as well as its co-purification in a complex with actin, emerin, nuclear myosin 1c, lamins A and B and SUN2,^{20,21} indicates that it is a significant component of the nucleoskeleton (Figure 8(b)).

In the cytoplasm, α II-spectrin interacts with β II-spectrin to form a heterodimer important in cytoskeletal structure and function.^{23–25,36} Though both the long form of β IV-spectrin, i.e., Sp β IV Σ 1, and β II-spectrin have been identified in the nucleus of nonerythroid cells,^{22–24,98} no definitive association between α II spectrin and these β -spectrins has been identified. As mentioned above, α II-spectrin's interaction with actin and protein 4.1 in the nucleus could be via an association with a β -spectrin, as it is in the cytoplasm (Figure 8(b)). Such an association could play an important role in nuclear architecture and structure and function of the nucleoskeleton. Both Sp β IV Σ 1 and β II-spectrin have sites that can bind actin and protein 4.1.^{23–25,100} α II-spectrin co-immunoprecipitates with Sp β IV and, though it associates mainly with the truncated Sp β IV Σ 5,¹⁹ it has some

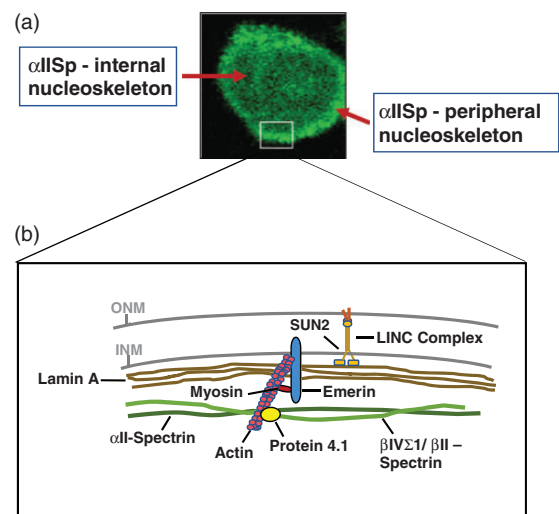


Figure 8. α IISp in normal human cells is present in both the peripheral and internal nucleoskeleton. (a) In normal human lymphoblastoid cells, using indirect immunofluorescence and staining with anti- α IISp, α IISp is observed in both the internal nucleoskeleton and the peripheral nucleoskeleton. (Frame reproduced from Zhang *et al.*⁶⁴ with permission from Wiley). (b) This is a diagram of the proposed interaction of α IISp with some of the proteins in the peripheral nucleoskeleton. α II-spectrin is proposed to interact with β -spectrin (β -II or β IV Σ 1) and form a cortical network with lamin A and actin that is anchored by emerin, nuclear myosin and protein 4.1 to the nuclear envelope^{7,19,20}. This is supported by studies which show that α IISp co-immunoprecipitates with lamin A, actin, protein 4.1, emerin and β IV-spectrin¹⁹ and co-purifies with lamin A, actin, emerin, nuclear myosin 1c and SUN2, a component of the LINC complex.^{20,21} β II-spectrin has been shown to be present in the nucleus^{101–104} and though it has not, as yet, been demonstrated to interact with α II-spectrin, it has been included in the diagram. Thus, α IISp may have an influence on the interaction of nuclear lamins with the LINC complex and may play a role in the biomechanical properties of the nucleus and mechanical coupling of the nucleus and cytoplasm in either undamaged and/or DNA damaged cells.

association with Sp β IV Σ 1 (unpublished data). Sp β IV Σ 1 is present in the nuclear matrix.¹⁰⁰ Thus, potentially, α II-spectrin could form an association with Sp β IV Σ 1 in the nucleus which would be important in its interaction with actin and protein 4.1. It could also potentially form such an interaction with β II-spectrin. Such interaction of α II-spectrin with either β IV-spectrin or β II-spectrin has been shown to occur in different locations in the cytoplasm in neurons.^{125–128} Thus, this type of interaction could potentially take place in the nucleus under different circumstances. The existence and nature of the interaction of α II-spectrin with these as well as other nucleoskeletal proteins remain a largely unexplored area which needs to be more fully investigated. It will be extremely important to determine this so that the functional significance a deficiency in α II spectrin has on these interactions may be determined.

Thus, α II Sp has both structural and non-structural roles in the nucleus. In its structural role, together with other nucleoskeletal proteins, it is thought to make an important contribution to nuclear architecture and the organization, stability, elasticity, and mechanical properties of the nucleus.^{7,20,30} In its non-structural role, it is best known for its involvement in DNA repair, where it plays an important role in the repair process during S-phase of the cell cycle, indicating its importance in DNA repair at the time of DNA replication.^{12–19} In addition, it could play a role in other functional activities in the nucleus such as chromatin remodeling, transcription, and RNA processing.¹⁹ Thus, deficiencies in α II Sp in a cell could have far reaching consequences and may impact a number of important cellular functions.

Additional potential roles for α II Sp in the nucleus

Another very interesting finding regarding α -spectrin function has been described by Goodman *et al.*^{40,129,130} They have demonstrated that in human erythrocytes, α -spectrin has chimeric E2/E3 ubiquitin conjugating/ligating enzymatic activity.^{39,129,130} Post-translational modification of proteins by ubiquitin involves a multi-enzymatic system consisting of: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin protein ligase (E3).^{131–134} Such modification can affect functional regulation of proteins, cellular regulatory mechanisms, and signal transduction and DNA repair pathways.^{40,131–134} In human erythrocytes, it has been demonstrated that α I-spectrin is monoubiquitinated at two sites by its own E2/E3 enzymatic activity.^{40,133,135} One of these sites, in the N-terminus, represents the location where α I-spectrin makes contact with β I-spectrin to form a heterodimer.^{133,135,136} Goodman *et al.* showed that ubiquitination of α I-spectrin does not regulate the rate of heterodimer formation; however, ubiquitination of α I-spectrin increases the rate of dissociation of actin and protein 4.1 from β I-spectrin.^{133,136,137} These studies are of interest since they indicate that ubiquitination of α I-spectrin could potentially affect cytoskeletal organization.

Whether, in non-erythroid cells, α II-spectrin could have similar E2/E3 ubiquitinating activity is a very interesting

question. Based on sequence similarity between erythroid and non-erythroid α -spectrin and conservation of sequences in these proteins throughout evolution, Goodman *et al.*⁴⁰ have suggested that α II-spectrin could serve as an E2/E3 ubiquitin conjugating/ligating enzyme in non-erythroid cells. They have shown that α SpII Σ 1 is ubiquitinated in hippocampal neurons, which supports this hypothesis.¹³⁸ Whether α II-spectrin in mammalian nuclei could also have E2/E3 chimeric enzyme activity, and whether it undergoes self-ubiquitination, is unknown. Also, whether it could potentially ubiquitinate other proteins in the nucleus, as has been shown for α 1-spectrin in erythroid cells,⁴⁰ has not been reported. We have shown that knockdown of α II Sp in normal human cells (to levels approximately 35–40% of normal) does not affect the monoubiquitination of FANCD2.⁶⁴ This indicates that α II Sp is not involved in the monoubiquitination of FANCD2 and that it acts downstream of monoubiquitinated FANCD2 in the initial steps of the ICL repair process.⁶⁴ Whether α II Sp has E2/E3 enzymatic activities that would act in the later steps in the repair process or could be involved in monoubiquitination of other structural or non-structural proteins in the nucleus is an area which certainly needs to be investigated.

Another possibility is that if α II Sp in the nucleus has E2/E3 enzymatic activity which can lead to its self-ubiquitination, this increased level of ubiquitination could potentially lead to decreased association of actin and protein 4.1 with β -spectrin, as has been shown to occur in erythroid cells.^{136,137} Increased self-ubiquitination activity, as well as factors affecting such an activity, could have a significant influence on nucleoskeletal structure and function in both mature normal human cells and in human cells during development. This raises a number of interesting questions regarding α II Sp's potential E2/E3 enzymatic activity and the effect this could have on nuclear architecture and on nuclear functional activity.

α II Sp's involvement in biomechanical properties of the nucleus

Biomechanical studies have shown that the nucleus displays complex mechanical properties which include compressibility and elasticity.^{7,22,105,120,139} These studies point to involvement of the nucleoskeleton and proteins associated with it in this process.^{3,22,105,139} Lamins in the nucleoskeleton have been shown to stabilize the nuclear architecture and influence nuclear responses to mechanical signals.^{3,100,120,139} They are the major mechanical elements of the nucleus and make a critical contribution to the mechanical stiffness of the nucleus.^{7,20,108,139} Lamins are important for mechanotransduction (i.e. translating mechanical changes or stimuli a cell receives into biochemical signals) and have an important role in mechanically coupling the cytoskeleton to the nucleoskeleton.^{3,7,111,119,120,139}

It has been proposed that α II Sp forms a network in the nucleus which has a mechanical role.^{7,20,22} Studies in which levels of α II-spectrin in the nucleus of HeLa cells were knocked-down to approximately 50% of those found in control cells have demonstrated that there is a loss in nuclear recovery from compression due to a reduction in elasticity

of the nucleus.²² This has been attributed to reduction in the α II-spectrin network in the nucleoskeleton which has a negative impact on the resilience of the nucleus to compression.²² Disruption of the α II-spectrin network thus causes a reduction in the elasticity of the nucleus but has no effect on the stiffness.²² These results importantly indicate that α II-spectrin has a mechanical role in the nucleus at the inner nuclear membrane where it contributes to the resilience of the nucleus and its recovery after compression, providing elasticity to the cell nucleus.²² Thus, within the nucleoskeleton, there are two important networks that provide mechanical stability to the nucleus: the lamin network, which makes important contributions to the mechanical stiffness of the nucleus, and the spectrin network, which is important for providing elasticity of the nucleus.^{7,20,22,111,139}

Mechanical coupling between the nucleus and cytoplasm is critical for cellular function and has been shown to involve LINC (links the nucleoskeleton and cytoskeleton) complexes which span the nuclear envelope.¹⁴⁰⁻¹⁴⁴ Lamin A can bind directly to LINC complex proteins SUN1 and SUN2.¹⁴³ Association of A-type lamins and emerin with the LINC complexes may help mediate mechanotransduction signaling and nucleoskeletal-cytoskeletal coupling.^{7,143} α II-Spectrin has been shown, in HeLa cell nuclei, to form a protein complex with emerin, actin, nuclear myosin, lamins A and B, and SUN2.^{20,21} It is possible that it could have an influence on the interaction of nuclear lamins with the LINC complex. α II-spectrin may be important in LINC complex mechanical coupling of the nucleoskeleton and cytoskeleton, in undamaged cells and/or in cells which have undergone DNA damage. These are studies which still need to be carried out and which could be of great functional importance. This is of particular interest since the LINC complex proteins, SUN1 and SUN2, have been shown to play a role in the DNA damage response, in addition to the diverse other functions in which they are involved.¹⁴⁵⁻¹⁴⁹ Studies suggest that the LINC complex promotes homologous recombination at sites of DSBs in DNA and in this way may also function in DNA inter-strand cross-link repair, where homologous recombination takes place in the latter steps of the repair process.^{147,149}

Thus, interactions between lamin A, actin, emerin, spectrin, protein 4.1, and nuclear myosin may be important for the normal biomechanical properties of the nucleoskeleton and for maintenance of tension, stiffness, and elasticity of the nuclear envelope.^{7,21,22} A deficiency in one or more of these proteins could thus affect the stability of the nuclear architecture and mechanotransduction through LINC complexes at the nuclear envelope.⁷ Whether such a deficiency would affect the biomechanical properties of the nucleoskeleton after cells are treated with a DNA damaging agent is a very intriguing question. Since α IISp plays a role in DNA repair, it will be important to determine whether loss or a deficiency of α IISp and the resulting diminished DNA repair has an effect on these biomechanical properties after DNA damage.

Effects of loss of α IISp on cellular morphology

α IISp in the cytoplasm has been shown to play a role in cellular morphology and its loss can lead to changes in cell shape, cell proliferation, cell adhesion, and cell spreading capabilities. We have shown that, after knock-down of α IISp in normal human lymphoblastoid cells to the levels found in FA cells, which are 35–40% of normal levels, these cells lose their pleomorphic shape; they are smaller and rounded with few if any pseudopodia, resembling FA lymphoblastoid cells in culture.¹⁵ These studies suggest a role for α IISp in cell structure. Similarly, studies on depletion of α II-spectrin in Jurkat T-cells have demonstrated that α II-spectrin is important for cell adhesion and formation of actin-rich lamellipodia and filopodia which are involved in immunological synapse formation upon T-cell activation.¹⁵⁰ Depletion of α II-spectrin leads to changes in the dynamics of the actin skeleton and decreased actin accumulation in areas of cell contact, indicating the important role of α II-spectrin in actin organization and function in the cytoskeleton.¹⁵⁰ In addition, studies have shown that, after knock-down of α II-spectrin by siRNA in a human melanoma cell line, the cells became rounded, decreased in size and had defects in cell proliferation, cell adhesion and spreading and displayed cell cycle arrest.³⁷ They also showed disorganization of the actin skeleton and loss of actin stress fibers.³⁷ These studies suggest that α II-spectrin may play a role in actin network formation.³⁷ Of interest, investigations on macrophages from *Fancc*^{-/-} mice have shown that these cells are round in shape, lack the multiple protrusions seen in wild type macrophages, and have deficiencies in cell migration.¹⁵¹ These cells showed impaired filamentous actin rearrangements.¹⁵¹ Since we have found that there is a deficiency in α IISp in bone marrow cells from *Fancc*^{-/-} mice (unpublished results), we have speculated that the morphological changes observed in macrophages from *Fancc*^{-/-} mice are due to reduced levels of α IISp in these cells which in turn leads to rearrangements of the actin filaments.¹⁵ These studies, collectively, suggest that α IISp plays a role in actin skeletal organization in the cytoplasm and that a deficiency in α IISp affects cellular morphology, in part through its effects on actin filament organization. Since we and others have shown that α IISp interacts with actin in the nucleus,^{7,19,20} it is possible that α IISp has a similar role in organization of actin in the nucleus, resulting in similar consequences when a deficiency in nuclear α IISp occurs. This is an exciting area of investigation that needs to be explored further.

Clinical significance of loss of α IISp

α IISp is an essential cellular protein. Total loss of α IISp leads to cell death.^{15,37,82,83} Thus, loss or dysfunction of α IISp in cells is of profound clinical relevance. In FA patient's cells, a deficiency in α IISp correlates with phenotypic changes occurring after exposure to DNA ICL agents (i.e. chromosomal aberrations, cell cycle defects, defects in repair of DNA ICLs, and telomere dysfunction).¹⁵⁻¹⁷ Our studies, which demonstrate that restoration of α IISp levels to

normal in FA cells corrects a number of the phenotypic deficiencies occurring after ICL damage, are of particular interest.¹⁶ In these studies, knockdown of μ -calpain led to an increase of α IISp to normal levels but had little effect on cell viability.¹⁶ Studies using a mouse model have shown that decreasing levels of μ -calpain by siRNA knockdown has no effect on development.¹⁵² Thus, developing methodologies that target μ -calpain and reduce its levels or activity in FA cells, so as to reduce cleavage of α IISp, could be of clinical importance, possibly in conjunction with other modalities, and suggests a potential new direction that could be explored for therapeutic intervention in FA.

Studies indicate that there may be a link between loss of α IISp and the pathogenesis of neoplastic bone marrow disorders such as leukemia.^{152,153} It has been shown that, in bone marrows examined from acute myeloid leukemia (AML) patients, 44% had a loss of α IISp, which suggests a possible role for α IISp in leukemogenesis.^{153,154} This is of particular interest since FA patients develop bone marrow failure and have a strong predisposition to develop AML.^{54,155} If α IISp loss plays a role in leukemogenesis, this could be of significant importance in the etiopathogenesis of these disorders.

Since α IISp is an important component of the nucleoskeleton and its levels are decreased in cells from FA patients,^{11,13,18} an extremely interesting question is what effect does a deficiency in α IISp have on various cellular processes such as development. In mammalian cells, α IISp is expressed throughout all stages of development and is critical in many developmental processes.^{38,156–158} Studies have shown that, in α IISp-spectrin knockout mice, embryos with a homozygous deletion of the *Spna2* gene (the gene coding for α IISp-spectrin) displayed prominent cardiac, craniofacial and neural tube abnormalities and died between day 12.5 and day 16.5.³⁸ Loss of α IISp-spectrin also reduced the steady state protein levels but not the transcriptional levels of β II- and β III-spectrin, leading to redistribution of both β II- and β III-spectrin as well as of ankyrin.³⁸ Cultured embryonic fibroblasts from *Spna2*^{-/-} mice displayed impaired growth and spreading and sparse lamellipodia which lacked cortical actin.³⁸ Thus, loss of α IISp-spectrin disturbs the levels and distribution of its associated proteins (i.e. β -spectrins, actin, and ankyrin).³⁸ These studies indicate that α IISp is required for the stability and organization of these proteins and for cell spreading, tissue patterning, and organ development in vertebrates.³⁸ They highlight the important effect α IISp has on cellular morphology and development.

FA patients show a number of congenital abnormalities, such as radial ray deformities, absent radii, urogenital malformations, renal anomalies, and ear malformations.¹⁵⁹ It may be hypothesized that a deficiency in α IISp, which has both structural and non-structural roles, may have an important influence on developmental processes and is a significant factor leading to many of the congenital abnormalities observed in FA patients. According to this hypothesis, a deficiency in α IISp in cells such as those from FA patients leads not only to defects in DNA repair and chromosome instability but also to development abnormalities;

these abnormalities may be due wholly or in part to loss of α IISp and its interaction with other nucleoskeletal proteins. Thus, development of technologies which restore levels of α IISp to normal in cells such as those from FA patients may potentially provide an important target for therapeutic regimes.

There is evidence that α II-spectrin plays critical roles in brain development and epileptic encephalopathy.^{128,160–162} Mutations in *SPTAN1*, the gene encoding α II-spectrin, have been shown to cause early infantile epileptic encephalopathy type 5 (EIEE5) leading to severe neurodevelopmental impairment and progressive brain atrophy.^{128,160–162} These mutations in *SPTAN1* lead to severely altered neuronal morphology.^{128,160} α II-spectrin is a critical structural protein in neurons.^{24,34,128,138,163} In EIEE5, the most severe mutations in *SPTAN1* lead to defects in binding of α II-spectrin to β -spectrins (II-IV).¹²⁸ Binding of α II-spectrin to β II or β IV spectrin depends upon their location in the neuron or the stage of development of the neuron.^{160,161,163} This binding is critical for neuronal development and structure.^{24,160,161} The importance of this interaction is clearly demonstrated in EIEE5 where deficiencies in this binding lead to severe neurological abnormalities.¹²⁸ Whether this type of interaction between α II-spectrin and β -spectrins also takes place in the nucleus, though this has been proposed, is not yet entirely clear. However, this raises the interesting possibility that a deficiency in interaction of α II-spectrin and β -spectrins in the nucleus could lead to identification of another group of pathological disorders.

As described above, a deficiency or loss of α II-spectrin in cells can be due to a number of different causes and can lead to a number of different pathological manifestations, depending on the disorder. In FA, for example, reduced levels of α II-spectrin in cells (35–40% of normal) are due to increased cleavage by μ -calpain at its cleavage site.¹⁶ In this disorder, it is the FA gene products, the FA proteins, which are proposed to play a role in maintaining the stability of α II-spectrin in the cell by limiting its cleavage by μ -calpain.¹⁶ This disorder is characterized by developmental abnormalities, bone marrow failure, a predisposition to cancer, and DNA repair defects.^{54–65,59–63} In another disorder, EIEE5, reduced levels of α II-spectrin are due to mutations in the α II-spectrin gene, *SPTAN1*, which leads to loss of α II-spectrin in neurons.^{128,160–162} This disorder is characterized by severe neurodevelopmental deficiencies and progressive brain atrophy.^{128,160–162} Thus, severe clinical pathologies are observed when there are losses in α II-spectrin due either to its increased breakdown or to its reduced expression in cells.

An important factor that needs to be kept in mind when examining cells for levels of α II-spectrin in the nucleus is that this is a labile protein. It can break down easily, especially when samples are being prepared for analysis, such as for Western blots.^{11,12} Thus, care needs to be taken in sample preparation. Further investigations into levels of α II-spectrin in various pathological disorders could thus potentially reveal that there is a loss of α II-spectrin, due either to its enhanced breakdown or to its decreased expression, which could provide important insights into the etiology of these disorders.

Conclusions and perspectives

α IISp, an essential cellular protein and an important component of the nucleoskeleton, has structural and non-structural functions, both of which are important. The best documented role of α IISp in human cell nuclei is in DNA repair.^{12–19} Our laboratory has shown that α IISp is critical for repair of ICLs both in genomic and, separately, in telomeric DNA and is necessary for telomere function and for chromosome stability after DNA ICL damage.^{11–19} We have proposed that α IISp functions as a scaffold aiding in the recruitment of repair proteins, such as XPF-ERCC1, to sites of damage and that it acts downstream from monoubiquitinated FANCD2.^{12,15,64} Based on our studies, we have hypothesized that, mechanistically, α IISp binds to DNA at sites of damage and recruits XPF-ERCC1 to these sites via FANCG, which acts as a link joining α IISp to XPF-ERCC1, enabling incisions to be made at the site of damage.^{32,74} Thus, α IISp contributes to an essential component of the ICL repair process, which involves a multitude of other proteins. We have proposed that FA proteins play an important role in maintaining the stability of α IISp in the cell and have suggested several mechanisms as to how this may occur.^{16,18} Additionally, α IISp's interactions with other proteins in the nucleus, such as those involved in other types of DNA repair, chromatin remodeling, transcription and RNA processing, indicate that it has a number of other non-structural roles in the nucleus.¹⁸

α IISp also has a structural role in the nucleus that has been far less extensively examined. It associates with the peripheral nucleoskeleton and co-immunoprecipitates and/or co-purifies with a number of the peripheral nucleoskeletal proteins.^{19–21} These include lamin, emerin, actin, protein 4.1, nuclear myosin, β IV-spectrin, and SUN proteins.^{19–21} Through its functional interactions with these other structural proteins, α IISp may have a significant role in the architecture and mechanical properties of the nucleus.^{7,20–22} It has been shown to make an important contribution to the resilience and elasticity of the nucleus.^{7,20,21} However, the effect DNA damage has on these functions has not yet been investigated and this clearly warrants further investigation.

The finding that α IISp has both structural and non-structural roles in the nucleus contributes to our understanding of the significance a loss in α IISp can have on cell function. In a disorder such as FA, in which we documented a marked deficiency in α IISp in the nucleus, the importance this deficiency has on the phenotypic characteristics observed is clearly demonstrated by studies which show that restoration of α IISp levels to normal corrects these defects.^{16,17} FA patients, however, also have a number of congenital abnormalities. An important question is whether a deficiency in α IISp in FA cells is related to these defects. In mouse models, loss of α IISp has been shown to lead to developmental anomalies.³⁸ It is thus possible that α IISp plays an important role in human development. A deficiency in α IISp in FA could be involved in the developmental anomalies observed, such as failure of digit formation and renal abnormalities. This is a totally unexplored area which would be exceedingly interesting to

investigate and could lead to determination of whether a deficiency in α IISp is an important factor in these developmental abnormalities.

Loss of α IISp could be of clinical significance in other disorders. An important question is whether such a loss plays a role in the pathogenesis of bone marrow diseases such as leukemia. This is of particular interest since a study has shown that there is a loss of α IISp in bone marrow from a sizable portion of AML patients.^{153,154} Other investigations have demonstrated that specific mutations in the *SPTAN1* gene coding for α II-spectrin, which affect the ability of α II-spectrin to bind to β -spectrins in neurons, lead to a group of infantile epileptic encephalopathies (EIEE5).^{128,160–162} These findings further confirm the importance of interaction of α II- and β -spectrins for neuronal cytoskeletal structure and function.^{128,160–162} It will be of considerable interest to determine whether such a relationship between these spectrins is also present in the nucleus and whether deficiencies in this interaction contribute to specific pathological conditions.

Thus, a deficiency in α IISp in cells has the potential to affect a number of different systems encompassing both structural and non-structural elements of cell function. Obtaining a better knowledge of α IISp's functional interaction with other nuclear proteins will increase our understanding of the clinical significance loss of α IISp has on critical cellular systems and enhance our understanding of the pathogenesis of disorders, such as FA and EIEE5, in which there is a deficiency in α IISp.

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