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REVIEW ARTICLE

SPINOPHILIN: A multiplayer tumor suppressor

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Abstract SPINOPHILIN (SPN, PPP1R9B or NEURABIN-2) is a multifunctional protein that regulates protein–protein interactions in different cell signaling pathways. SPN is also one of the regulatory subunits of protein phosphatase 1 (PP1), implicated in the dephosphorylation of retinoblastoma protein (pRB) during cell cycle. The *SPN* gene has been described as a tumor suppressor in different human tumor contexts, in which low levels of SPN are correlated with a higher grade and worse prognosis. In addition, mutations of the SPN protein have been reported in human tumors. Recently, an oncogenic mutation of SPN, A566V, was described, which affects both the SPN–PP1 interaction and the phosphatase activity of the holoenzyme, and promotes p53-dependent tumorigenesis by increasing the cancer stem cell (CSC) pool in breast tumors. Thus, the loss or mutation of SPN could be late events that promotes tumor progression by increasing the CSC pool and, eventually, the malignant behavior of the tumor.

SPINOPHILIN

SPINOPHILIN (SPN), also known as PPP1R9B and NEURABIN-2, is a protein that interacts with protein phosphatase 1 (PP1) and α-actin. This protein is expressed in many tissues, especially in the dendritic spines of the central nervous system, where the majority of the excitatory impulses from the nervous system are received.[1–4] Two different groups independently described the SPN protein for the first time. Allen et al carried out a double hybrid assay to identify regulatory proteins of the catalytic subunit of PP1 and

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identified a protein of 817 amino acids named SPINOPHILIN due to its location in dendritic spines.[1] In addition, Satoh et al identified a new actin-binding protein named NEURABIN-2 because of its similarity to NEURABIN-1, which ultimately turned out to be SPN.[2]

The *SPINOPHILIN* gene (*SPN* or *PPP1R9B*) has 10 exons and is located on chromosome 17 at the 17q21.33 position, a chromosomal region frequently associated with microsatellite instability and loss of heterozygosity.[3–11] In addition, this region contains a high density of well-known tumor suppressor genes, such as *BRCA1* and *NME1*; putative genes, such as *JUP* and *PROHIBITIN*; and some unidentified candidate genes located close to the *BRCA1* locus.[3–11] Most of the studies that have examined region 17q21 have focused on the *BRCA1* gene, which shows loss of heterozygosity with different frequencies depending on the type and status of the tumor.[3–11] However, other studies explored the genetic association of breast and ovarian cancer with the 17q21 region and suggested the existence of a new tumor suppressor gene located close to *BRCA1*. Ultimately, *SPN* was identified as that gene.[3–11]

Structure of the SPN protein

SPN is a multifunctional protein whose structure suggests that it functions as a chaperone or a scaffold protein by recruiting different proteins into different cell signaling pathways.[4] To allow protein–protein interactions, SPN presents different domains and motifs: two *actin-binding domains* (ABD1 and ABD2) located at positions 1–154 and 164–282, respectively; an *SH3 domain* (SH3D) consisting of three proline-rich regions located at positions 8–14, 137–143 and 281–287; a *receptor-binding domain* located at positions 151–444 that interacts with seven transmembrane domain receptors; a *PP1 binding domain* located at positions 417–494; an alpha helical motif that forms coiled coils called *leucine/isoleucine zipper (LIZ)* located at positions 485–510 that allows protein–protein interactions, especially targeting kinases and phosphatases to some ion channels; and a *PDZ domain* at positions 492–583 that allows binding to certain proteins at its C-terminus and a *coiled-coil domain* at position 664–814 that allows SPN to form homo- or heterodimers (Fig. 1).[2–4,12]

SPN presents many phosphorylated residues for different protein kinases among its structure. Protein kinase A (PKA) phosphorylates SPN in S97 and S177, calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylates SPN in S100 and S116, cyclin-dependent protein kinase-5 (CDK5) in S17 and mitogen-activated protein kinase-1 (MAPK1 or ERK2) in S15 and S205 (Fig. 1).[4,13–15]

SPN is located mainly in the cytoplasm and in the plasma membrane of cells, although reports have shown that SPN could also be expressed in the nucleus.[4,16] Moreover, the expression of SPN is enriched in synapses and in cell–cell adhesion sites based on E-cadherin.[2,4]

SPN as a PP1 regulatory protein

PP1 is a ubiquitously expressed serine–threonine phosphatase protein involved in the regulation of many cellular processes, such as neuronal signaling, protein synthesis, muscle contraction, transcription, apoptosis and cell cycle progression.[17–20] In mammals, three genes (*PPP1CA*, *PPP1CB*, and *PPP1CC*) encode four isoforms of the catalytic subunit of PP1: PP1α, PP1β, PP1γ1 and PP1γ2. These isoforms are expressed in all tissues and cell compartments, except PP1γ2, which is only expressed in testes. The isoforms share a large part of their sequence, with 93% shared between PP1γ1 and PP1γ2 and 85% shared between PP1β and PP1γ2, although the N-terminus and the C-terminus present greater differences.[19,21–27] All the isoforms are found in the nucleus, although PP1β and PP1γ show a special accumulation in the nucleolus.[19] Furthermore, the expression of the different isoforms varies according to the tissue; for example, in the brain, PP1β is the most abundant isoform in the body of neurons or soma, while PP1α and PP1γ1 are concentrated in dendritic spines and in the synapse.[26,28]

Despite the numerous functions in which it is involved, PP1 by itself does not have substrate specificity and needs interaction with multiple regulatory proteins to achieve this specificity. Thus, the catalytic subunit of PP1 (PP1C) can bind and form different holoenzymes with multiple regulatory proteins that direct PP1 to specific substrates in specific cell locations. Three different PP1C isoforms have been described: PPP1CA, PPP1CB and PPP1CC.[19,21,22,29–31]

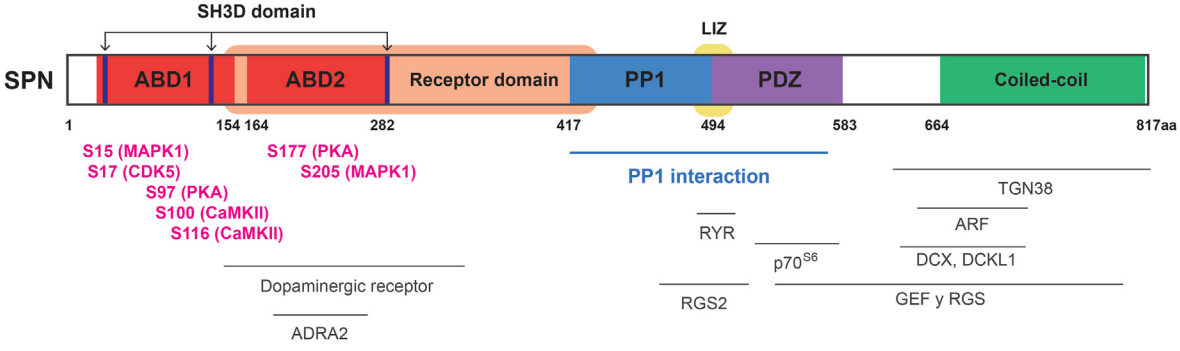

Figure 1 Structure of the SPN protein. Scheme of the domains and motifs of SPN, some of its most important interactions with other proteins and the most relevant phosphorylations (highlighted in pink) with their respective kinases/phosphatases. Figure is adapted from references[3] and[4].

The catalytic active site of PP1 contains two metal ions and is located at the intersection of three substrate-binding sites named hydrophobic, acidic and C-terminal grooves.[22] PP1 regulatory proteins determine the ability of PP1 to bind to different substrates. Approximately 90% of PP1 regulatory proteins bind PP1 through the PP1 binding motif (RVxF motif), whose consensus sequence is [K/R] [R/K] [V/I] [x] [F/W] and where "x" can be any residue except F, I, M, Y, D or P.[18,22,29,31] The interaction through this RVxF motif is necessary for the binding of the PP1 regulatory protein, although it does not affect the enzymatic activity of PP1 since it is located 20Å from the active site.[21,22,29,30] Furthermore, this interaction is unique to each PP1 regulatory protein since the binding site of different proteins is the same or overlaps. Thus, mutations in the RVxF motif would prevent the binding of a PP1 regulatory protein but would not affect the binding of the substrate or other holoenzymes.[18] In addition to the RVxF motif, other interactions have been described to stabilize PP1 binding and allow for modulation of the activity and specificity of the holoenzyme, such as the SILK and MyPhoNE motifs.[19,21,22,29–31] For all these reasons, the development of specific inhibitors or activators that compromise the interaction of PP1 with a PP1 regulatory protein and/or the regulation of the holoenzyme could have great potential for the treatment of numerous diseases, such as those related to the nervous system or cancer.[22,25]

PP1 regulatory proteins direct PP1 to different substrates to perform specific functions.[21,22,29,30] These proteins are dynamic and unstructured since they lack 3D folding when they are not bound to PP1, which allows them to bind PP1 over an extensive interaction surface.[21,22,29–31] The PP1 binding domain of SPN (residues 417–494) is unstructured and highly dynamic when SPN is not bound to PP1. However, when both proteins interact, this domain folds completely to achieve a single stable conformation. Despite being unstructured, this domain is fully functional and interacts strongly with PP1, being necessary and sufficient to fully interact with PP1.[21,22,29,30,32]

The SPN–PP1 interaction occurs not only through the RVxF motif but also by forming multiple interactions with different regions of PP1, including part of the C-terminus of PP1.[29] In total, the region of SPN that interacts with PP1 consists of residues 417–583 (Fig. 2A). Therefore, the dynamic nature of SPN in its unbound form is essential to form such binding with PP1.[29] The SPN–PP1 interaction can be divided into 4 regions (Fig. 2B, C).[30] Region 1 is the RVxF motif (RKIHF, residues 447–451) and interacts with the RVxF binding groove of PP1. Residue H450 plays an important role in the interaction with PP1 since it forms a hydrogen bridge with residue T288 of PP1 (Fig. 2D).[30] Region 2 consists of residues 430–434 and 456–460, which fold to form two beta sheets (β-sheets of SPN 1/2) that extend the beta sheet of PP1 (β-sheets of PP1 14/13/2/3/4)

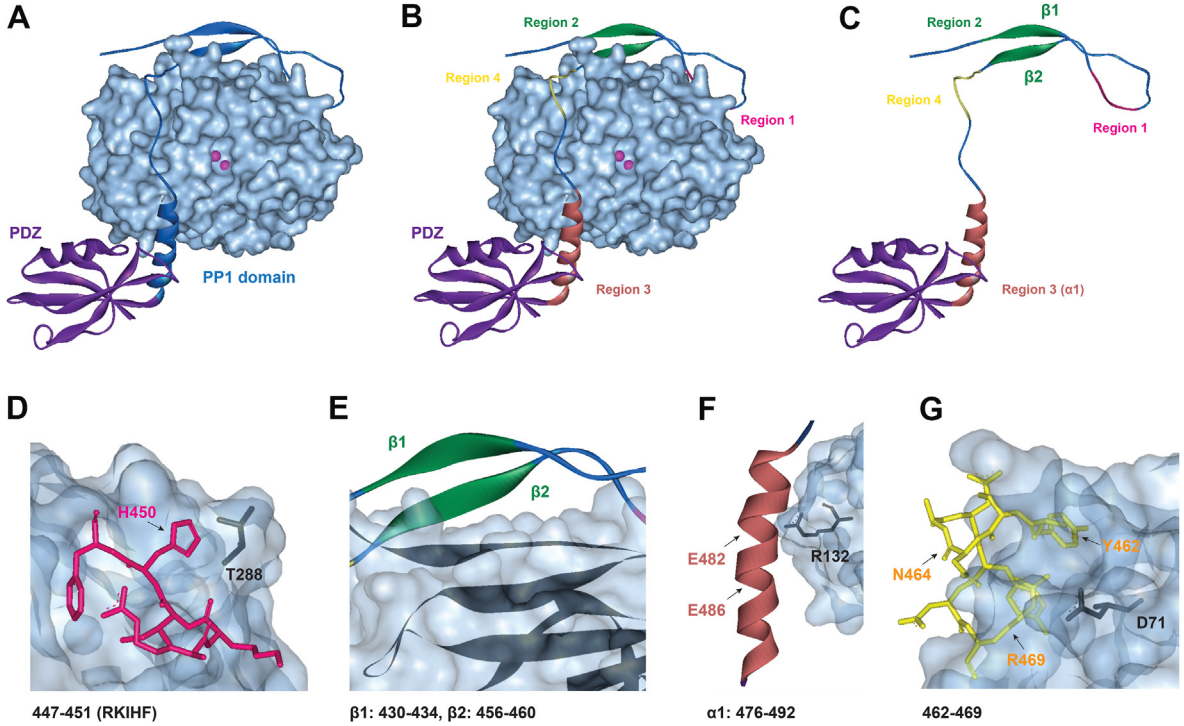

Figure 2 SPN–PP1 interaction. **(A)** Representation of SPN–PP1 interaction. The structures of SPN and PP1 are partially represented: SPN residues 424–583 and PP1 residues 7–300. The molecule represented in light blue corresponds to the structure of PP1. Two manganese ions are represented in pink, indicating the active site of PP1. The other molecule is SPN, in which the PP1 binding domain (417–494) is highlighted in dark blue and the PDZ domain (492–583) is highlighted in purple. **(B, C)** Representation of the different regions or SPN–PP1 interaction. **(D)** Region 1 (RVxF motif): residues 447–451 of SPN are highlighted in pink and residue T288 of PP1 is highlighted in black. **(E)** Region 2: the two beta sheets of SPN (green) extend the beta sheet of PP1 (black). **(F)** Region 3 (alpha helix) is represented in red, and residue R132 of PP1 is represented in black. **(G)** Region 4 is represented in yellow, highlighting residues Y462, N464 and R469 that interact with residue D71 of PP1 in black. Figure is adapted from reference[29].

on a seven-strand beta sheet (Fig. 2E).[30] Region 3 includes residues 476—492, which fold into a 4-turn alpha helix to form electrostatic and hydrophobic interactions with the surface of PP1 and are adjacent to the hydrophobic and C-terminal grooves. Specifically, residues E482 and E486 of SPN make contact with residue R132 of PP1. Indeed, the alpha helix and beta sheets were not observed if SPN was not bound to PP1 (Fig. 2F).[30] Finally, residues 462—469 form region 4, which binds to a substantial part of the C-terminal groove of PP1. The center of the interaction is formed by residue R469, which forms hydrophobic and electrostatic interactions with PP1 through residue D71 of PP1 and residues Y462 and N464 of SPN (Fig. 2G).[30] Therefore, SPN binds to a large part of the C-terminal groove of PP1, thereby blocking access to other substrates of PP1. Although the SPN—PP1 interaction is fairly extensive, it does not alter the active site or the hydrophobic and acidic grooves of the enzyme PP1.[30]

The mutations that affect the SPN—PP1 interaction the most are found in the residues of the RVxF motif, especially in the F451 residue, and in the C-terminal binding motif (residues Y467 and R469), although other important residues are F459, T461, Y462, and N464.[21,22,29,30] Recently, a mutation in the PDZ domain of SPN, A566V, was described with oncogenic potential.[33] Therefore, the critical residues for PP1 binding are located not only in the RVxF motif but also in the region that binds to the C-terminal groove of PP1, which is necessary to direct the substrate specificity of PP1.[21,22,29,30]

On the other hand, inhibitor-2 (I-2) is a specific inhibitor of PP1 that is ubiquitously expressed and has a role in cell cycle regulation because it translocates to the nucleus during S phase and mitosis.[22] I-2 binds PP1 through the SILK motif (residues 12—17), the RVxF motif (residues 44—56) and residues 130—169, which form an alpha helix.[21] PP1 is known to bind both a regulatory protein and an inhibitor to form a heterotrimer so that both proteins collaborate and make the regulation of PP1 more complex, which is the case for the complex formed by PP1, SPN and I-2 (PSI). SPN and I-2 colocalize in dendritic spines and actin-rich adherent junctions so that the PSI complex could have a role in cytoskeletal rearrangement and neuronal signaling.[21,22,34] Although both proteins form unique interactions with PP1, they also share some binding sites, such as the RVxF motif.[21,22] When the PSI complex is formed, structural rearrangements occur in SPN and I-2 proteins but not in PP1. The RVxF motif of SPN interacts with PP1 more strongly than that of I-2; therefore, in the formation of the PSI complex, the RVxF motif of I-2 is released, and only the motif of SPN binds PP1.[21,22] For that reason, the SILK motif of I-2 plays a greater role in the formation of the PSI complex. Therefore, other motifs and domains beyond the RVxF motif and the catalytic site of PP1 allow the interaction with PP1, and they could be important in regulating PP1 and generating new and different holoenzymes.[21,22]

Other interactions of SPN

SPN allows the interaction between different proteins; to date, more than 30 proteins that interact with SPN have been described (Fig. 1).[4] In this review, we only describe those interactions that may contribute to the tumor suppression role of SPN beyond PP1, collaborating or not with PP1.

NEURABIN-I

SPN interacts with its homologous NEURABIN-I (also known as PPP1R9A), and they can form homo- or heterodimeric complexes between them. These two proteins share some biochemical properties, have 80% homology in their sequence and perform similar functions.[4,17,18,26,28] For example, both of them are regulatory proteins of protein phosphatase 1 (PP1), and its interaction occurs through the PP1 binding domain that is present. However, they also have independent functions and are regulated differently by phosphorylation.[4,26,28] Indeed, while SPN is ubiquitously expressed, NEURABIN-I is only expressed in neural tissue.[1,2,4] In addition, NEURABIN-I and SPN have more preference for PP1γ1 and PP1α isoforms than for PP1β, to which only NEURABIN-I is able to bind. Surprisingly, none of them associates with protein phosphatase 2 (PP2A).[26,28]

DOUBLECORTIN (DCX)

SPN also interacts with DOUBLECORTIN (DCX), a cytoplasmic protein that mediates neuronal migration during the development of the cerebral cortex. DCX is a microtubule-associated protein that binds tubulin and microtubules through tandem repeats at the N-terminus, inducing their polymerization.[4,35] Its structure presents two DCX domains at the N-terminus and a Ser/Pro-rich domain at the C-terminus: the first DCX domain binds microtubules, while the second is able to bind both microtubules and unpolymerized tubulin. Specifically, DCX binding to microtubules is negatively regulated by phosphorylation on serines 47 and 297.[36] The interaction between DCX and SPN occurs through the coiled coil domain of SPN (residues 649—696) and a region of DCX located between the end of the second DCX repeat and the beginning of the Ser/Pro-rich domain (residues 246—303) (Fig. 1).[4,35,36]

DCX and SPN are coexpressed in many common brain regions, especially in the telencephalon. They are also expressed in the soma of neurons and in neurites, and the colocalization of these two proteins has been reported in hippocampal neurons.[37] DCX binds to both microtubules and actin, while SPN only binds to actin. Since both microtubules and actin filaments are involved in neuronal migration, both cytoskeletons could be connected through a heterodimer formed by DCX—SPN.[36,37] The distribution of DCX between both cytoskeletons could be regulated by phosphorylation by SPN/PP1; so that when DCX is phosphorylated, it does not bind to microtubules but binds to actin through SPN interaction.[36,37] Therefore, the binding of DCX to actin is regulated by SPN and both proteins cooperate to link microtubules and actin, contributing to neuronal migration.[36,37]

DCX is phosphorylated by different kinases, such as protein kinase A (PKA), microtubule affinity regulating kinase (MARK), cyclin-dependent kinase 5 (CDK5) and c-Jun N-terminal kinase (JNK).[38,39] While CDK5 phosphorylation

prevents DCX binding to microtubules, the others are involved in neuronal migration and the growth of the cones of differentiated neurons. On the other hand, DCX is dephosphorylated by PP1 through interaction with SPN, and this dephosphorylation is specific for JNK phosphorylated residues (T331 and S334).[38,39] Phosphorylated DCX binds to actin through SPN, and when it is dephosphorylated by PP1, DCX stops binding actin (and SPN) to rejoin microtubules. Therefore, the regulation of the state of phosphorylation of DCX determines whether it binds to microtubules (dephosphorylated) or to actin (phosphorylated).[38,39] In addition, SPN also facilitates dephosphorylation of DCX at Ser297 by PP1 in the wrist of the axons, a residue phosphorylated by CDK5 kinase. Thus, the DCX—SPN interaction as well as CDK5 phosphorylation/PP1 dephosphorylation at Ser297 of DCX are necessary for the maintenance of microtubules during neurite growth.[35]

DOUBLECORTIN-LIKE KINASE-1 (DCLK1)

SPN also binds to a DCX-related protein, doublecortin-like protein kinase-1 (DCLK1/DCAMKL1), in the coiled coil domain. This protein presents a DCX-homologous N-terminal and a C-terminal serine/threonine-dependent protein kinase domain with calcium/calmodulin-dependent protein kinase activity.[4,37] SPN binds to DCLK1 through the DCX homology domain. Therefore, the patterns of expression of DCX and DCLK1 in the brain are very similar.[4,37]

DCLK1 was first reported as a potential marker of stem-like cells of the small intestine[40] and is now an established tumor stem cell marker in different types of tumors, including colon, breast, pancreas, kidney, liver and digestive tract tumors.[41—44] DCLK1 regulates multiple biological processes in cancer, such as tumor growth, pluripotency, invasion, metastasis and epithelial-to-mesenchymal transition (EMT).[41,43,45] The overexpression of DCLK1 results in the acquisition of self-renewal capacity in differentiated hepatocytes and hepatoma cells by activating the noncanonical β-catenin signaling pathway,[42] whereas its down-regulation in esophageal squamous-cell carcinomas inhibits tumor progression by suppressing the β-catenin/c-Myc pathways.[45] Other studies also associate DCLK1 with the PI3K pathway, acting as a potent oncogene to accelerate invasion, metastasis and EMT of colorectal cancer cells.[45] DCLK1 is a promising therapeutic target since it represents a specific CSC marker in different types of tumors.[41,44] However, the mechanism through which DCLK1 is involved in tumorigenesis is not completely understood. The interaction between SPN and DCLK1 may also be relevant in tumorigenesis, although more studies are needed in this field.

Guanine exchange factors (GEF)

SPN interacts with some guanine exchange factors (GEFs) that regulate G-protein signaling proteins (RGS) associated with the GTPase RAC, such as TIAM1, KALIRIN-7 and RASGFR1, through its PDZ domain and part of the coiled coil domain (residues 444—817).[4] SPN binds to TIAM1, promoting its localization in the plasma membrane and preventing other RAC effectors from binding, such as PAK1.[4,46] SPN binding to TIAM1 also enhances its ability to activate $p70^{S6}$ over other RAC effectors and recruit additional regulatory proteins into the signaling complex.[4,46] SPN also interacts through its PDZ domain with the protein kinase $p70^{S6}$, a mitogen-activated kinase that controls protein translation and cell proliferation.[4] Indeed, the binding of PP1 and $p70^{S6}$ to SPN is mutually exclusive.[4]

On the other hand, SPN interacts with the dopaminergic receptor D2, which activates protein kinase $p70^{S6}$ through the PI3K/mTOR pathway.[4] Therefore, SPN could be involved in $p70^{S6}$-dependent proliferation by two different mechanisms. Since PP1 is a negative regulator of TIAM1 and the binding of PP1 and $p70^{S6}$ to SPN is mutually exclusive, PP1 could serve as a mechanism to inhibit such activation.[4]

$p14^{ARF}$

Another important interaction of SPN is the tumor suppressor ARF ($p14^{ARF}$), a nucleolar protein with which SPN interacts through the coiled coil domain (residues 605—726). This protein is encoded by an alternative reading frame of the p16 gene (*CDKN2A*), and it functions as an inhibitor of MDM2, the protein that induces the degradation of p53.[47—51] ARF hijacks MDM2 to prevent the translocation of p53 from the nucleus to the cytoplasm, inhibiting its degradation. The stabilization of p53 by ARF induces cell cycle arrest; therefore, SPN and ARF could have an additive effect in suppressing cell growth.[4,16,52] In hepatocellular carcinoma, the impairment of the SPN—ARF interaction caused by decreased SPN expression could influence ARF-mediated tumor suppressive functions.[53] Other studies using *Spn*-null MEFs showed increased p53 activity dependent on $p19^{ARF}$; however, no physical interaction between SPN and $p19^{ARF}$ was detected.[52] Therefore, more studies are needed to clarify the role of the interaction of SPN and ARF.

SPN is a multifunctional protein that interacts with many different proteins in different cell signaling pathways and contributes to several cellular processes that, when deregulated, could promote tumor progression.

Role of SPN in the cell cycle

PP1 indirectly controls cell cycle progression by modulating the phosphorylation status of key cell-regulatory proteins, including retinoblastoma protein (pRB).[17—20] The three isoforms of PP1 bind pRB in the same way since the interaction region is conserved and all of them have the ability to dephosphorylate pRB; however, they present different activities in the different phases of the cycle.[27,54—56] PP1α is the main isoform that controls pRB during G1 and in the G1/S transition.[17,57] During mitosis, all isoforms are phosphorylated and inactivated because an increase in the phosphorylation of PP1α occurs at serine residues and of PP1β and PP1γ1 occurs at threonine residues. As cells exit mitosis, PP1α and PP1β activity increases while PP1γ1 activity remains low because it remains phosphorylated.[58,59] In fact, PP1β is the most active isoform during mitosis, although this activity does not persist during G1.[27,54,56,58—60] Therefore, the dephosphorylation of pRB is regulated in a sequential and temporal manner and the three isoforms of PP1 form

different holoenzymes with different regulatory proteins that have different preferences for phosphorylation sites, which also occurs with phosphorylation by CDK/cyclin complexes.[54,56,60,61]

On the other hand, when pRB is dephosphorylated during the middle of the mitosis phase, PP1 is phosphorylated and inactive; therefore, different holoenzymes of PP1 may occur during the cell cycle to control the dephosphorylation of pRB.[23,55,57,59,61,62] Specifically, PP1β has been reported to bind to a regulatory protein of approximately 110 kDa when it binds to pRB during mitosis.[23,62,63] Phosphatase nuclear targeting subunit (PNUTS) is a PP1 inhibitory protein with an important role in controlling PP1 activity during mitosis by inhibiting pRB dephosphorylation. However, PNUTS is only associated with a small proportion of PP1; therefore, other proteins beyond PNUTS must regulate PP1 during the cell cycle, such as SPN.[64] In fact, PNUTS and SPN bind PP1 in different regions without overlapping.[65] PNUTS is a context-dependent PP1 regulatory protein, and the role of SPN in PP1 regulation and pocket protein dephosphorylation might also be dependent on the context regarding either the cell cycle or subcellular localization.[66] In addition, pRB could function as a substrate or as a regulatory protein of PP1 since different subpopulations of pRB perform different functions depending on the type of phosphorylation.[66] Therefore, whether the dephosphorylation of proteins by PP1 in mitosis and in G1 occurs through a single mechanism or if different substrates are recognized by different holoenzymes must be determined.

The study of PP1 regulatory proteins involved in the cell cycle is very important since mutations in the catalytic subunit or the regulatory subunit of PP1 that prevent binding to pRB will promote phosphorylation of pRB and, eventually, cell transformation.[57,62,63,66,67] Furthermore, the interaction of PP1 with the regulatory protein and/or the regulation of the holoenzyme could represent a new therapeutic target for the treatment of diseases such as cancer.[22,25] Previous studies have shown that SPN has an important role in the regulation of PP1 and the dephosphorylation of pRB during the cell cycle; therefore, mutations in this protein could be of great interest in the initiation and progression of tumorigenesis.[52,68–70]

SPN is one of the regulatory subunits of the catalytic subunit of PP1α (PPP1CA), which is involved in both pRB and p53 dephosphorylation.[33,69,71] Loss of SPN has been reported to induce a proliferative response by reducing PPP1CA levels and increasing hyperphosphorylated and inactive pRB levels. In turn, this induces an increase in p53 activity through the ARF protein, producing neutralization of the proliferative response. However, the loss of SPN is frequently associated with p53 mutations; therefore, in the absence of p53, the loss or reduction of SPN levels produces an increase in cell proliferation and the tumorigenic properties of the cells are enhanced.[3,52] Indeed, not only is the loss of SPN associated with p53 mutations but also SPN mutations.[33] In a previous study, 50% of tumors with a SPN mutation also presented an inactivating mutation in p53, whereas those tumors with wild-type p53 carried other alterations capable of inactivating the p53 pathway, such as MDM2 amplification, deletion or methylation of CDKN2A (p14ARF), unbalanced NOTCH pathway, and noncoding specific microARNs.[33,72] The recently described oncogenic SPN-A566V mutation also demonstrated this p53 mutation-dependent effect in tumorigenesis by using an immortalized nontumorigenic cell line of epithelial breast tissue that expresses wild-type p53, MCF10A. The cells that carry SPN-A566V and p53-R175H mutations formed an increasing number of colonies and grew faster than cells with only SPN-A566V. Subsequently, the oncogenic effect of SPN-A566V depending on p53 mutations was corroborated using two p53-mutated breast cancer cell lines. Therefore, a functional relationship occurs between SPN and p53 inactivation so that SPN mutation alone is not able to initiate tumorigenesis, although it is a late event that promotes tumor progression and aggressiveness.[3,33,52,68,73,74] (Fig. 3).

The mutant SPN-A566V has been described to interact strongly with PP1α and PP1γ but not with PP1β, as previously described for wild-type SPN.[26,33] Meanwhile, PP1 binds P-pRB from the end of mitosis to the middle of G1,[60,66,75,76] and SPN interacts specifically with both total and phosphorylated pRB at Ser807/811, two of the preferred PP1 dephosphorylation sites.[25,33] The mutation SPN-A566V does not affect the interaction of SPN and pRB, although the holoenzyme PP1-SPN-A566V has a lower capacity to dephosphorylate P-pRB during G0/G1 transition and the G1 phase.[33] SPN-A566V may also induce a change in the localization of PP1, thus avoiding its translocation to the nucleus and partially preventing its activity on some substrates, such as pRB.[33] Therefore, the PP1-SPN holoenzyme seems to regulate the dephosphorylation of pRB exclusively during the G0/G1 transition and at the end of G1.[33] This holoenzyme does not act during the G2/M transition or the mitosis phase, when PP1β is the most active isoform and with which SPN does not interact.[33] Instead, PP1β could bind to a different PP1 regulatory protein during mitosis, although it remains unidentified.[23,62,63]

Pocket proteins (pRB, p107 and p130) are a family of very similar proteins that share some biochemical properties and some functions[56,77–79] and collaborate in different phases of cell cycle regulation.[77,80–82] The heterodimer SPN/PP1 is able to bind and dephosphorylate P-p107 at least at Ser975 and P-p130 at Ser672.[33] Although another phosphatase (such as PP2A) may dephosphorylate P-p107 and P-p130 in other contexts, the PP1–SPN holoenzyme is not exclusive to P-pRB but acts over all pocket family proteins.[33] Therefore, different holoenzymes could be involved in the sequential control of pocket protein dephosphorylation during cell cycle progression, and each holoenzyme might have distinct specificity to different phosphorylated residues, such as CDK/cyclin complexes; therefore, initial dephosphorylation would be necessary to induce a conformational change before any other holoenzyme gains access to different residues.[33,60]

SPN as a tumor suppressor

The locus of *SPN* is located in a chromosomal region associated with microsatellite instability, loss of heterozygosity and a high density of well-known tumor suppressor genes, such as *BRCA1*.[3,5–11] The loss of heterozygosity (LOH) in the 17q21 region has been reported in different tumors, such as breast, ovarian, lung, prostate, colorectal, gastric, renal and lung cancer.[3,5–11] Several studies

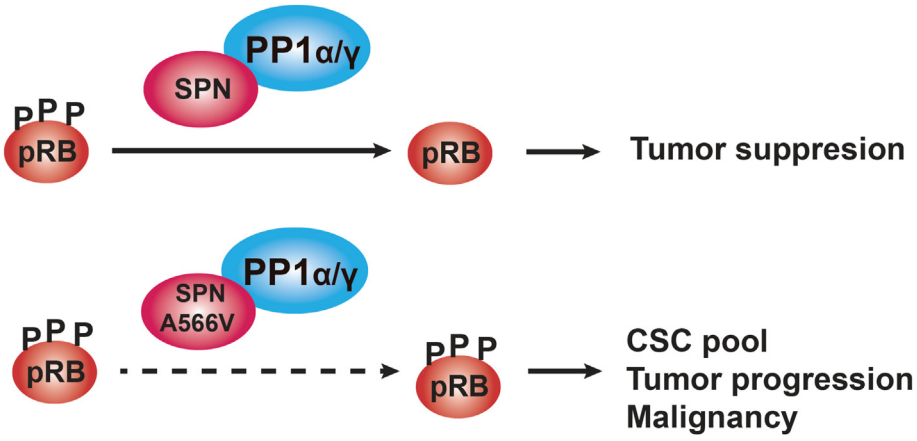

Figure 3 Scheme of the inhibition of SPN-A566V mutation on pRB dephosphorylation.

have suggested the existence of a new tumor suppressor gene located in the 17q21 region[3,5]; therefore, a LOH mapping was performed in this region on primary lung carcinoma using different polymorphic markers.[11] The D17S588 marker presented the highest value, with a loss of 53%. This marker was located in the *SPN* locus, and *SPN* was suggested as this new gene.[11]

To support the idea that *SPN* is a tumor suppressor gene, *in vivo* studies were performed using Spn-knockout mice.[68,83] The absence of Spn decreased the life expectancy of mice, increased the number of spontaneous tumors such as lymphomas and increased cell proliferation of certain tissues, such as the breast ducts. Surprisingly, $Spn^{-/-}$ mice did not express Spn in the mammary ducts, and both $Spn^{+/-}$ and $Spn^{-/-}$ mice showed more ramifications in this tissue.[68] Moreover, using p53-knockout mice, the combination of the loss of Spn and p53 increased preneoplastic lesions in the mammary glands.[68] Loss of Spn is thought to increase the p53 response in a similar way to that of senescence oncogene-induced senescence.[3,68] Thus, once spontaneous tumors appear and p53 is lost, the loss of Spn increases their aggressiveness. Therefore, the combination of the absence of Spn and the loss of p53 function promotes tumorigenicity *in vivo*.[3,68]

Currently, *SPN* has been described as a tumor suppressor gene in different contexts in human tumors. SPN levels are decreased in renal carcinomas, lung adenocarcinomas and tumors of the central nervous system, and low SPN levels correlate with a higher grade in ovarian carcinoma and chronic myeloid leukemia.[3,74] *SPN* has also been proposed as a tumor suppressor gene in head and neck carcinoma and in hepatocellular carcinoma, where patients with low levels of SPN expression had a worse prognosis.[53,84] Specifically, *SPN* has an important role in tumorigenesis as a tumor suppressor gene in the following types of cancer.

Lung cancer

A study in lung cancer showed that SPN is lost in 20% of tumors, while 38% of them presented low levels of SPN.[74] In addition, decreased SPN levels correlated with a higher grade of malignancy, a less differentiated phenotype, poor prognosis and p53 mutations.[74] In this study, different lung cancer cell lines with or without p53 mutations were used, demonstrating the role of *SPN* as a tumor suppressor gene dependent on the absence of p53.[74] This evidence correlated with previous studies that suggested a strong correlation between p53 mutations and the loss of the *SPN* locus.[52,68] Genetic studies have shown that *SPN* can be lost by heterozygous or homozygous deletion, either alone or in tandem with other tumor suppressor genes located at the 17q21 locus. Analysis of miRNAs in a series of lung tumors showed that miRNA106a* targeting SPN is overexpressed in some patients, correlating with decreased SPN levels. Therefore, miRNA106* overexpression is one of the possible mechanisms but not the only one, which may explain the downregulation of SPN in malignant lung tumors.[74]

Other studies have corroborated that SPN has prognostic and predictive value in lung cancer since the downregulation of this gene together with p53 mutations are associated with worse survival. This gene showed increased methylation in tumor samples in comparison to non-tumor samples, another possible mechanism implicated in the regulation of SPN expression levels.[70] Furthermore, the decrease in SPN levels is greater in squamous cell carcinoma than in adenocarcinoma, being the first more aggressive.[70] A correlation is observed between the decrease in SPN levels and low levels of the three catalytic subunits of PP1, and this combination is associated with a worse prognosis in squamous cell carcinoma. The SPN/PP1C ratio could serve as a response biomarker due to its prognostic and predictive value in this type of tumor.[70] Indeed, a direct correlation was observed between the SPN/PP1C ratio and the response to different drugs commonly used in the clinic, such as oxaliplatin and bortezomib; therefore, the SPN/PP1C ratio could also be used as a therapy response marker.[70]

Colorectal and gastric carcinoma

The 17q21 region is lost in a high percentage of colorectal and gastric carcinomas, and approximately 25% of colorectal, 35% of gastric and 15% of intestinal carcinomas have low levels of SPN.[3,73] Furthermore, this gene has prognostic and predictive value in advanced stages of colorectal carcinoma since stage III patients with low levels of SPN

showed worse survival and relapse.[73,85] Colorectal cell lines with low levels of SPN have increased expression of E2F-1, a well-known driving factor for cellular growth, and are more resistant to 5-fluorouracil treatment.[85] These cells were able to form a higher number and larger primary and secondary tumorspheres and showed an increase in CD133+ cells, a marker of cancer stem cells previously reported for colorectal cells.[85] Therefore, the loss of SPN produces an increase in the stemness properties of the cells, which could explain why tumors with low levels of SPN showed a worse prognosis since the poor response to chemotherapy and relapse are associated with a great number of cancer stem cells.[69,86] However, a functional correlation between SPN and p53 has not been observed in colorectal carcinoma.[73,85] Since 45% of colorectal carcinomas present a KRAS mutation in the early stages of the tumor and the loss of SPN is a late event that promotes tumor progression, in the presence of KRAS mutations, p53 would not need to be mutated.[73] Therefore, the correlation between SPN and p53 would be dependent on the molecular context.[73,85]

Glioblastoma

In glioblastoma, SPN controls tumor invasion by regulating invadopodia, actin-rich protrusions of the plasma membrane that are associated with the degradation of the extracellular matrix during invasion and metastasis,[87] which seems to occur through the depolymerization of actin by the SPN—PP1—DCX complex.[88] In glioma tumor cells that overexpress DCX, reports have indicated that DCX can only interact with SPN when it is phosphorylated, and this phosphorylation is JNK dependent.[88] Specifically, the region involved in binding DCX to SPN and PP1 is the KVRF motif and the JNK sites of DCX. Furthermore, the interaction between DCX and SPN depends on the phosphorylation of DCX, allowing the formation of the DCX—SPN—PP1 complex.[88]

DCX blocks mitosis in the G2/M phase, thereby preventing microtubule polymerization and mitotic spindle formation through SPN interactions.[89] This process is known as mitotic catastrophe, a type of apoptosis characterized by the formation of non-viable, multinucleated cells with condensed chromosomes.[89] Thus, the double overexpression of DCX and SPN produces cycle arrest in G2/M phase and apoptosis. When DCX is phosphorylated, it interacts with SPN and PP1 in the cytosol, thereby preventing PP1 from transporting to the nucleus to bind and dephosphorylate kinesin-13, a microtubule-depolymerizing protein.[89] In this way, kinesin-13 is phosphorylated and active and produces microtubule depolymerization, preventing the formation of the mitotic spindle and, therefore, stopping cells in G2-M phase.[89] In addition, phosphorylated DCX inhibits invasion in glioma cells by inducing depolymerization of actin through the complex that forms with SPN and PP1.[89]

On the other hand, DCX has been shown to prevent self-renewal of brain cancer stem cells and to promote differentiation and apoptosis with the help of SPN.[90] The phosphorylation of DCX by JNK induces the formation of the PP1—DCX complex and prevents the binding of PP1 to caspase-3 and consequently its dephosphorylation. Thus, phosphorylated caspase-3 is active and promotes apoptosis.[90]

Furthermore, SPN has been shown to directly bind to the cytoplasmic domain of β8 integrin in glioblastoma cells.[87] Downregulating SPN leads to an increase in the ability of glioblastoma cells to invade by modulating invadopodia dynamics.[87] This formation and dissolution of invadopodia regulated by SPN is dependent, at least in part, on correct activation of the GTPase RAC1.[87] Glioblastoma cells that lack SPN show diminished RAC1 activities, increased numbers of invadopodia, and enhanced extracellular matrix degradation, indicating that SPN is a critical adhesion regulatory protein essential for cell invasion in the brain microenvironment.[87]

Therefore, SPN plays an important role in glioma depending on its interaction with other proteins beyond PP1, such as DCX[88] and β8 integrin.[87]

Breast cancer

SPN plays an important role as a tumor suppressor gene in breast cancer.[69,91] SPN levels are reduced or lost in approximately 15% of breast tumors, and a correlation is observed with a higher histological grade, a less differentiated phenotype and worse survival. Indeed, aggressive tumors, such as estrogen-receptor-negative tumors and triple-negative or basal tumors, presented lower levels of SPN than luminal tumors.[69,91] In addition, both SPN and p53 are lost in triple-negative tumors, and this combination makes tumors more aggressive.[92] Downregulation of SPN in breast cancer cell lines increases some tumorigenic properties, such as cell proliferation, anchorage-independent growth and migration, while overexpression of this gene produces the opposite effect.[69,91] Moreover, the downregulation of SPN also induces an increase in the stemness properties of the cells, such as the formation of tumorspheres and the expression of CSC markers (*NANOG*, *OCT4*, *SOX2* and *KLF4*).[69] In breast cancer, $CD44^+/CD24^-$ cells have been proposed to be cancer-initiating cells with stem cell properties; therefore, these markers would serve to identify cancer stem cells.[93] Enrichment in $CD44^+/CD24^-$ cells has been observed in tumors with lower levels of SPN and in breast cancer cell lines with downregulated SPN.[69] In addition, low levels of SPN have also been associated with cancer stem cells in other types of tumors, such as colon cancer.[85] These effects seem to depend on PP1 since its downregulation in breast cancer cell lines produced the same effect.[69] The loss of SPN in breast cancer produces an increase in the stemness properties of the cells, which may explain the poor response to chemotherapy of those tumors.[69,86]

Since the role of SPN as a tumor suppressor gene is dependent on PP1 activity, the overexpression of different mutations in the region of interaction between SPN and PP1 was evaluated. Thirty-nine mutations were identified in this region in different human tumors.[33] An oncogenic mutation of SPN, SPN-A566V, which affects the PP1 phosphatase activity, especially over the pocket proteins, was identified and characterized.[33] The expression of the SPN-A566V mutation in breast cancer cell lines induces an increase in the tumorigenic and stemness properties of the cells.[33] Similar to the loss of SPN, the effect of this mutation was dependent on p53 mutations.[33,69] Thus, cells with SPN-

A566V and mutated p53 grew faster and formed larger tumors and showed a potentiation of the stemness phenotype, such as an increase in the formation of tumorspheres, a higher percentage of $CD44^+$ $CD24^-$ cells and the expression of some CSC markers.[33] Therefore, as previously described for the loss of SPN, mutations in this gene are a late event that depends on p53 inactivation to initiate tumorigenesis, promoting tumor progression and aggressiveness by increasing stemness and the pool of $CD44^+$ $CD24^-$ cells.[33]

A connection between the cell cycle and stem cell biology was also proposed via SPN/PP1/pocket proteins.[33] Cells that overexpress SPN-A566V have high levels of P-pRB, P-p107 and partially P-p130 during the G0/G1 transition and at the end of G1, which could mean that they have a shorter G1 phase to proliferate more rapidly.[33] Recently, pRB was reported to be directly involved in the transcriptional regulation of the pluripotency genes *OCT4* and *SOX2*.[94] SPN-A566V cells have high levels of P-pRB, NANOG, OCT4 and SOX2.[33] When pRB is dephosphorylated and active, the OCT4 and SOX2 promoters are inhibited[95]; thus, P-pRB may promote OCT4/SOX2 expression in SPN-A566V cells, which in turn induces NANOG.[95,96] On the other hand, OCT4 regulates the self-renewal and differentiation of embryonic stem cells and controls the cell cycle by increasing CDK/cyclin levels during the G1 phase and by preventing pRB dephosphorylation by PP1.[97–99] Therefore, further studies are needed to clarify whether the PP1—SPN holoenzyme plays any role in the OCT4/pRB self-regulatory circuit.

Conclusions and perspectives

The *SPN* gene has been described as a tumor suppressor in different contexts in human tumors, such as lung cancer, colorectal and gastric carcinoma, glioblastoma and breast cancer. Loss and mutations of SPN are implicated in a p53-dependent tumorigenesis by increasing the CSC pool. The role of SPN in tumorigenesis has been associated with the interaction of PP1 and the regulation of the PP1—SPN holoenzyme, especially in the dephosphorylation of pRB during the cell cycle. However, since SPN mediates many protein—protein interactions in different cell signaling pathways, other mechanisms implicated in tumorigenesis beyond PP1, such as DCX or ARF, should be explored. SPN is an interesting tumor suppressor with an important predictive and prognostic value in different types of cancer, which makes it a potential biomarker of response to antitumoral therapy.

Author contributions

EMVS and AC contributed to the design, writing and editing of this manuscript. All authors have read and agreed to the published version of the manuscript.

Conflict of interests

The authors declare no conflict of interests.

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