Spinophilin acts as a tumor suppressor by regulating Rb phosphorylation

Irene Ferrer[1,4], Carmen Blanco-Aparicio[1], Sandra Peregrino[1], Marta Cañamero[2], Jesús Fominaya[1], Yolanda Cecilia[2], Matilde lleonart[3], Javier Hernandez-Losa[3], Santiago Ramon y Cajal[3], Amancio Carnero[1,4]*

1)Experimental Therapeutics programme, 2) Biotechnology programme, Spanish National Cancer Research Centre, Madrid, Spain
3) Pathology Department, Fundacio´ Institut de Recerca Hospital Vall d'Hebron, Barcelona, Spain
4) present address: Institut d'Investigació Biomédica de Bellvitge-IDIBELL
5) present address: Instituto de Biomedicina de Sevilla, Consejo Superior de Investigaciones Cientificas, Sevilla, Spain

*) Correpondence to Amancio Carnero, Instituto de Biomedicina de Sevilla/HUVR, Consejo Superior de Investigaciones Cientificas, Campus HUVR, Edificio IBIS, Avda. Manuel Siurot s/n. 41013, Sevilla, Spain. acarnero@us.es; acarnero@ibis-sevilla.es Tel:34955923111

Author e-mail addresses: Irene Ferrer (ireneferrersan@yahoo.es); Carmen Blanco-Aparicio (cblanco@cnio.es), Sandra Peregrino (speregrino@cnio.es), Marta Cañamero (mcanamero@cnio.es), Yolanda Cecilia (ycecilia@cnio.es), Jesús Fominaya (Jfominaya@cnio.es), Matilde lleonart (matildelleonart@yahoo.es), Javier Hernandez-Losa(jahernan@vhebron.net), Santiago Ramon y Cajal(sramon@vhebron.net), Amancio Carnero (acarnero@us.es)

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Abbreviations

Spn, Spinophilin, PPP1R9B

SPN, Spinophilin protein

PP1a, phosphatase 1A

PPP1CA, catalytic subunit of PP1a

Rb, retinoblastoma protein

Running title: **Spinophilin is a tumor suppressor by regulating Rb**

ABSTRACT

The scaffold protein Spinophilin (SPN) is a regulatory subunit of phosphatase1a located at 17q21.33. This region is frequently associated with microsatellite instability and LOH containing a relatively high density of known tumor suppressor genes, including *BRCA1*. Several linkage studies have suggested the existence of an unknown tumor suppressor gene distal to *BRCA1*. *Spn* may be this gene but the mechanism through this gene make its contribution to cancer has not been described. In this study, we aimed to determine how loss of *Spn* may contribute to tumorigenesis. We explored the contribution of SPN to PP1a-mediated Rb regulation. We found that the loss of *Spn* downregulated PPP1CA and PP1a activity, resulting in a high level of phosphorylated Rb, and increased ARF and p53 activity. However, in the absence of p53, reduced levels of SPN enhanced the tumorigenic potential of the cells. Furthermore, the ectopic expression of SPN in human tumor cells greatly reduced cell growth. Taken together, our results demonstrate that the loss of *Spn* induces a proliferative response by increasing Rb phosphorylation, which in turn activates p53, thereby, neutralizing the proliferative response. We suggest that *Spn* may be the tumor suppressor gene located at 17q21.33 acting through Rb regulation.

INTRODUCTION

The *Spinophilin* (*Spn, Neurabin II, PPP1R9B*) locus is located on chromosome 17 at position 17q21 which is in a cytogenetic area frequently associated with microsatellite instability and loss of heterozygosity (LOH). This region has a relatively high density of tumor suppressor genes, including known (e.g., *BRCA1* and *NME1*), putative (e.g., *JUP* and *Prohibitin*), and unidentified candidate tumor suppressor genes that are located distal to the *BRCA1* locus. Most of the studies examining the 17q21 region have focused on *BRCA1*, which exhibits LOH at a variable frequency depending on the type and stage of the tumor, and as such, the 17q21 region has been extensively studied in breast carcinomas [1-3]. However, some studies have suggested the presence of an unknown tumor suppressor gene in the area that includes the *Spn* locus. LOH at 17q21.3 involving the *BRCA1* locus has been observed in breast, ovarian, prostate, colorectal, gastric, renal, and lung carcinomas, as well as in salivary gland carcinosarcomas, an extremely aggressive neoplasm. This region contains both *NME1* and *Spn*, which are only 1 Mb apart. Extensive LOH mapping in primary lung carcinomas [4] using 15 highly polymorphic markers revealed the highest LOH value (53% loss) with the D17S588 marker, which is located within the *Spn* locus. However, neighboring tumor suppressor genes, including *BRCA1*, were not significantly affected (6–13% LOH). Furthermore, previous classic cytogenetic studies that investigated the genetic association of breast and ovarian cancers with the 17q region have suggested the presence of a tumor suppressor gene located distal to *BRCA1* [5-7]. The maximum LOD scores obtained for D17S588 were 5.44 in an Edinburgh study of 15 families [8] and 21.68 in an extensive analysis of 271 families with breast and breast/ovarian cancer [9-10], indicating the importance of this region in cancer pathology. Another study examining the correlation between p53 abnormalities and allelic loss of *BRCA1*, *BRCA2*, and adjacent loci in breast cancer found a strong correlation between *p53* mutations and the specific loss of the *Spn* locus [1, 11].

Although these studies suggest the existence of an unknown tumor suppressor in this region, no biological function for *Spn* has been reported that would explain the benefits that a tumor cell might gain by losing this gene. Furthermore, the correlation between *p53* mutations and the specific loss of the *Spn* locus is not understood. The focus of this

study was to determine how the loss of *Spn* may contribute to tumorigenesis and the role of *p53* mutations in this process.

Two independent laboratories have shown that SPN interacts with protein phosphatase 1 (PP1) and F-actin [12-13]. SPN contains a number of distinct domains that govern protein-protein interactions, including two F-actin domains, three potential Src homology 3 domains, a receptor and a PP1-binding domain, a PDZ domain, three coiled-coil domains, and a potential leucine/isoleucine zipper motif [14]. While more than 30 SPN-binding partners have been discovered, including cytoskeletal and cell adhesion molecules, enzymes, guanine nucleotide exchange factors (GEFs), regulators of G-protein signaling, membrane receptors, ion channels, and the tumor suppressor ARF [14], the physiological relevance of some of these interactions remains undetermined. The structure of SPN suggests that it functions as a multifunctional scaffold protein that regulates both membrane and cytoskeletal processes. SPN mediates important functions in the nervous system, where it has been implicated in regulating spine morphology and density, synaptic plasticity, and neuronal migration. SPN also regulates seven-transmembrane receptors and may link these receptors to intracellular mitogenic signaling events that are dependent on $p70^{S6}$ kinase and Rac G protein-GEF. Importantly, a role for SPN in cell growth has also been demonstrated, and this effect is enhanced by the interaction between SPN and ARF [15]. Another SPN-interacting molecule is doublecortin, an actin-binding protein with an established role in the subcellular targeting of PP1 [13, 16-17]. SPN enhances PP1-mediated dephosphorylation of Ser297 in doublecortin [18] and its localization to the cytosol [19-20]. One of the most important PP1 target proteins is Rb, the phosphorylated product of the *retinoblastoma* gene that is essential in cell cycle regulation [21]. The targeting of Rb by PP1 contributes to the dephosphorylation and subsequent activation of Rb, shutting down the G1/S phases of the cell cycle [21-22]. Furthermore, the PP1a-mediated dephosphorylation of Rb contributes to the senescent phenotype induced by oncogenic Ras [23] and the constitutive inactivation of Rb by maintaining highly phosphorylated Rb contributes to cell cycle deregulation and tumorigenesis [24-26]. Therefore, SPN may contribute to tumorigenesis by regulating the functions of PP1 and/or Rb.

This study aimed to elucidate the molecular mechanisms that could mediate *Spn* loss-induced tumorigenesis, focusing on the SPN-PP1-Rb complex. We found that the loss

of *Spn* correlated with a reduced level of PPP1CA, which in turn maintained an elevated level of Rb. This effect contributed to a functional increase in p53 activity through ARF. However, in the absence of p53, SPN enhanced the tumorigenic properties of the cells.

RESULTS

The absence of *Spn* contributes to Rb deregulation by maintaining a low PPP1CA level and PP1 phosphatase activity.

Because SPN binds to PPP1CA, thereby contributing to the regulation of PP1a activity, we first measured the effect of loss of *Spn* on PPP1CA expression. We generated mouse embryonic fibroblasts (MEFs) from *Spn* KO mice (Spn(-/-)) [18] and measured their PPP1CA level. An analysis of more than 20 pairs of wild-type (WT) and Spn (-/-) MEF clones demonstrated that Spn (-/-) cells contained 40% less PPP1CA protein (Figure 1A and 1B). Despite the parallel isolation of the MEF clones (i.e., all *Spn*-null clones were compared to their WT sibling clones at the same growth stage and passage), their PPP1CA levels seemed to be heterogeneous, perhaps due to variations in other PPP1CA-binding proteins. To determine whether the loss of *Spn* also affected the PPP1CA level under stress conditions, we expressed oncogenic Ras in the cells; this has previously been reported to increase the PPP1CA level [23, 27]. The expression of oncogenic Ras induced a 50% increase in the PPP1CA level in the WT MEFs, but not in the Spn (-/-) MEFs (Figure 1C and 1D). We also observed that the expression of oncogenic Ras resulted in a similar increase in the SPN protein level in the WT MEFs (Figure 1C), suggesting that SPN and PPP1CA may be co-regulated and may both contribute to the regulation of PP1 activity. To further confirm this hypothesis, we aimed to determine whether the loss of SPN altered PP1a activity as a result of the reduced PPP1CA level. We quantified the PP1 activity as the difference in activity measured following treatment with 2.5 nM and 2.5 μM okadaic acid [28]. We observed that PP1 phosphatase activity also decreased in the *Spn*-null cells in parallel with PPP1CA protein level, and this decrease was not reversed following the expression of oncogenic Ras (Figure 1E and 1F). To explore the mechanisms underlying the reduced PPP1CA level in *Spn*-null cells, we performed similar experiments in the presence of the proteasome inhibitor MG132. In the absence of proteasome-mediated degradation, the PPP1CA expression level and PP1 activity did not decrease in the absence of *Spn*

(Figure 1G and 1H). These results suggest that SPN may regulate the stability of PPP1CA, thereby regulating the activity of PP1a.

To evaluate whether the decrease in PP1 activity has physiological significance, we measured the extent of Rb phosphorylation in the absence of SPN. Furthermore, because Rb phosphorylation controls S phase entry, we also examined whether the Rb phosphorylation correlated with alterations in the cell cycle. The cells were serum starved for 24 hrs to induce Rb dephosphorylation and growth arrest. The cells were then restimulated with 10% serum, and Rb phosphorylation was analyzed at different time points. In the WT cells, Rb was fully dephosphorylated up to 8 hrs after restimulation (Figure 2A and B), correlating with entry into S phase (Figure 2C). The *Spn*-null MEFs exhibited low, but detectable, levels of pRb even in the absence of serum, indicating reduced PP1 activity. Furthermore, 16 hr post-stimulation, the level of pRb doubled in the Spn-null MEFs (Figure 2A and B), correlating with the earlier entry of the cells into S phase (Figure 2C). In the absence of SPN, we detected both increased basal expression and stronger induction of Cyclin A, a transcriptional target of E2F1 activation, following serum restimulation (Figure 2D and 2E), confirming the increased pRb phosphorylation. The higher level of pRB in the Spn-null MEFs also contributed to increased apoptosis in the Spn(-/-) MEFs upon p53 activation by DNA-damaging agents (Supplementary Figure 1).

These results clearly demonstrate that, in the absence of SPN, PP1 exhibits reduced phosphatase activity, resulting in an elevated level of pRb.

The absence of *Spn* contributes to genetic alterations during MEF immortalization.

Several alterations occur during MEF immortalization, resulting in the release from senescence. These mutations tend to eliminate the G1-phase arrest imposed by senescence. Most commonly, MEF immortalization results from the loss of the *INK4a* locus or *p53* mutations [29-31]. The *INK4a* locus contains two genes, *p16INK4a* [32], an inhibitor of CDKs that is upregulated during senescence and contributes to Rb dephosphorylation [33], and *p19ARF*, an MDM2 regulator that contributes to p53 activation [34-35].

We reasoned that if the *Spn*-null MEFs exhibited alterations in the Rb pathway, there would be no selective pressure to mutate *p16INK4*. To confirm this hypothesis, we generated multiple clones from WT (+/+), heterozygous (+/-), and *Spn*-null (-/-) MEFs and immortalized these cells using standard 3T3 protocols. The loss of *Spn* did not preclude the cells from entering senescence, and this response occurred with kinetics similar to WT cells [36]. However, *Spn* deficiency did affect the pattern of genetic alterations that occurred during MEF immortalization. Approximately 30% of the WT immortalized MEFs clones exhibited decreased p16INK4 expression, and 50% of the clones carried p53 mutations. However, neither the heterozygous nor the *Spn*-null (-/-) MEFs lost p16INK4a expression (Figure 3). All Spn-null (-/-) MEF clones were immortalized through mutations in p53 (Figure 3). All of the data regarding p53 mutations that were detected based on the stabilization of p53 were confirmed by sequencing *p53* mRNA (data not shown).

The lack of *Spn* contributes to increased p53 activity.

The lack of SPN seemed to promote p53 mutations in MEFs during immortalization, suggesting a functional relationship between SPN and p53. In addition, it has been shown that low levels of Rb activity activate the E2F family of transcription factors, which in turn activate p19ARF, thereby contributing to p53 upregulation [37-39,40]. To explore this possibility, we first tested the effect of shRNA-mediated SPN silencing in cells expressing the Val135 thermosensitive p53 mutant (p53(-/-) ts cells) [23] (Figure 4A). Switching the cultures to 32°C resulted in growth arrest, allowing only a small number of colonies to escape the arrest and to grow slowly. Silencing of SPN enhanced the observed p53-induced growth arrest in these cells. We then determined the p53 level in *Spn*-null MEFs. The *Spn*-null MEFs exhibited a slightly elevated level of p53, resulting in an elevated level of its downstream target p21waf1 (Figure 4B). Following induction of DNA damage with etoposide, a topoisomerase inhibitor, the increase in the p53 and p21waf1 levels were more significant in *Spn*-null MEFs when compared to WT cells (Figure 4B).

Next, we aimed to determine the importance of the increased p53 levels induced by the loss of *Spn* to the tumor physiology. 3-MC is a mutagen that induces tumor formation in a p53 mutation–dependent manner [41]. The addition of 3-MC enhanced the p53 response more significantly in Spn-null MEFs than in WT MEFs (Figure 4C). As expected, the

loss of *Spn* delayed the onset of tumorigenesis, increasing the survival of WT mice (Figure 4D). However, in mice with reduced p53 levels (i.e., heterozygous for p53), the loss of *Spn* did not increase the survival rate (Figure 4E). We could not perform these experiments in p53-null;Spn(-/-) or p53-null;Spn(+/-) mice due to their rapid death days after birth (data not shown). Taken together, our data suggest that the loss of *Spn* increases the activity of p53.

The increased p53 activity observed in *Spn*-null MEFs seemed to be dependent on PPP1CA, as PPP1CA overexpression in *Spn*-null MEFs abolished this increase (Figure 5A and supplementary figure 3). Ectopic expression of PPP1CA induced growth arrest. Among the resistant clones the doubling time was similar to parental cells expressing only vector (data not shown). In these clones we measured PPP1CA levels (supplementary figure 4) and we found that PPP1CA levels are only slightly increased. In these cells DNA-damage treatments induce p53 stabilization but the enhancement of the signal observed in the absence of Spn is loss (Figure 5A). In addition, the increased p53 activity was also dependent on p19ARF. First, *Spn*-null MEFs exhibited increased p19ARF activation following expression of oncogenic Ras when compared to WT MEFs (Figure 5B). Furthermore, *Spn*-null MEFs expressing a p19ARF-specific shRNA did not demonstrate enhanced p53 activation following expression of oncogenic Ras when compared to the controls (Figure 5C and 5D), confirming that p19ARF mediates the increased p53 levels observed in *Spn*-null MEFs. However, we were unable to detect a physical interaction between SPN and p19ARF (Figure 5E and data not shown).

Our overall interpretation of these results is that the loss of *Spn* leads to Rb inactivation and the further sequential activation of E2F, ARF, and p53. Therefore, p53 plays rate-limiting, pivotal tumor suppressor role and must be mutated when SPN is non-functional in order for cells to become transformed.

The absence of Spn enhances the tumorigenic properties of p53-null cells.

Next, we aimed to determine the effects of p53 deficiency on *Spn*-null cells. To this end, we measured their ability to potentiate the p53-null phenotype. First, we grew MEFs expressing the thermosensitive p53 mutant (p53(-/-)ts) at 39°C, thereby inactivating p53. Under these conditions, the shRNA-mediated silencing of SPN increased cell colony formation (Figure 6A). Additionally, the loss of SPN also enhanced the growth

of the MEFs (Figure 6B) by decreasing doubling time, and their growth recovery after serum starvation and restimulation (Figure 6C). Finally, the transfection of MEFs with the *Ras* oncogene or a combination of *E1A* and *Ras* induced an increase in both the number and size of the foci in *Spn*-null MEFs compared to mock-transfected MEFs (Figure 6D). These results confirm that the loss of SPN in p53-deficient cells enhances the tumorigenic potential of the cells.

The overexpression of SPN reduces tumor cell growth.

Because reduced SPN levels increased the malignant potential of the tumor cells, we determined if SPN overexpression might affect the malignant behavior of tumor cells. To evaluate the effect of SPN overexpression, we transfected tumor cells with *Spn* cDNA. Overexpression of SPN significantly reduced the colony numbers in all of the tested cell lines (Figure 6E). This growth inhibitory effect is similar to the growth inhibition described for PPP1CA overexpression [23] and occurred in all tested cell lines regardless of their p53 and pRb status (Supplementary Table 1).

DISCUSSION

Spn has been proposed to be a tumor suppressor because of its association with the LOH detected in genomic linkage studies and because loss of *Spn* migth contribute to lung tumorigenesis [42]. However, it is not clear how the loss of *Spn* might affect cellular behavior and contribute to tumorigenesis. In this study, we demonstrate that the loss of *Spn* affected the behavior of Rb through its ability to regulate the PPP1CA level and PP1a activity. The loss of *Spn* resulted in Rb inactivation and the subsequent activation of E2F, ARF, and p53. However, in the absence of p53, the elevated pRb level enhanced the tumorigenic potential of the cells (Supplementary Figure 2). In line with this behavior, SPN has been shown to bind to the PP1-doublecortin complex, inducing its dephosphorylation [43] and inhibiting anchorage-independent growth in glioma cells [44-46]. In contrast, doublecortin-mediated growth repression is lost in the absence of *Spn*.

SPN appears to function as a classic scaffolding protein with no intrinsic enzymatic activity. SPN binds both PPP1CA and PPP1CC, but only marginally binds to PPP1CB [47]. SPN selectively interacts with PPP1CC in the spinal cord, and it has been suggested that this binding is at least in part responsible for the enrichment of PPP1CC at synapses

[48]. Similarly, the binding of SPN to PPP1CA results in partial coupled regulation between SPN and PP1a, as lower levels of SPN led to decreased PPP1CA levels and PP1 activity. It has been recently shown that SPN is unstructured in its unbound form and binds PP1 through a folding-upon-binding mechanism, blocking one of the three putative substrate binding sites of PP1 without altering the active site [49].

PP1 is one of the key eukaryotic serine/threonine phosphatases involved in mitotic dephosphorylation of both Rb and specific residues of p53 [50]. Our data suggest that the mild p53 activation observed in the absence of *Spn* is related to enhanced Rb phosphorylation and activation of E2F and p19ARF because eliminating p19ARF blocked the enhanced p53 activation induced by oncogenic stress (Figure 5). It is interesting to remark the increase in p19ARF observed in *Spn* null compared to WT MEFs (Fig 5B). This increase might be dependent on E2F1 activation by pRb phosphorylation, since ARF is a target of E2F1 transcription target [51]. Unfortunately, we do not currently understand why *p19ARF* is not deleted with the same frequency that *p53* is mutated in *Spn*-null MEFs. We can only speculate that the *INK4* locus contributes to senescence through both *p19ARF* and *p16INK4a* gene activation[52]. p16INK4a contributes to senescence through Rb, therefore alleviating the Rb tension (by *Spn* loss), reducing the requirement for *INK4* deletion.

However, similar to PPP1CA overexpression [21,23], SPN overexpression resulted in growth inhibition in culture, independently of the status of Rb and p53. This may be due to PP1-target proteins other than Rb, whose phosphorylation is thought to enable cells to replicate DNA, such as DNApolα or TopoII [53,54]. It is also possible that the other pocket proteins, p130 or p107, are involved in pRb null cells response to PPP1CA overexpression since these proteins may have partially redundant control of cell cycle [55]. However, complete loss of PP1a is also deleterious for the cells [21], and only partial loss of activity has been associated with tumorigenesis [23]. PP1 does not only regulate the cell cycle, and Rb is not its only substrate. Therefore, SPN loss would be expected to have pleiotropic effects that are not solely related to Rb, but which may be equally important for tumour cell growth/survival[54].

The combination of *Spn* loss and p53 deficiency resulted in greatly enhanced tumorigenic properties in the cells. These results can be extended to a mouse model: we have shown that Spn KO mice exhibit increased cellular proliferation in the mammary

ducts, which translates to an increase in benign mammary lesions. In addition, the loss of *Spn* in combination with mutant p53 resulted in a large increase in the number of mammary carcinomas, confirming the functional relationship between p53 and SPN [36]. Our data provide a functional explanation to several cancer studies that found a strong correlation between p53 mutations and the specific loss of the *Spn* locus (47.1 % LOH) [1, 11,42]. Again, SPN may be involved in tumorigenesis by functioning, in association with the loss of p53 activity, as a tumor suppressor.

SPN is a regulator of PP1a, and our data strongly argue in favor of PP1a as an important tumor suppressor. The downregulation of PPP1CA, the catalytic subunit of PP1α, has been shown to maintain the hyperphosphorylated state of Rb, allowing cell growth [23]. *PPP1CA* has been mapped to chromosome 11q13 [56], and translocations involving breakpoints at 11q13 have been observed in lymphomas, chronic B cell lymphocytic leukemia, and multiple myeloma [57-58]. Results from the analysis of human solid tumors suggest that one *PPP1CA* allele may be lost in a high proportion of carcinomas, such as kidney and colorectal cancer [23].

In summary, our data demonstrate that the scaffold protein SPN is important for the correct regulation of PP1α and Rb and that its absence may contribute to tumorigenesis in the absence of p53 *in vivo*. Therefore, the loss of *Spn* may induce a proliferative response by increasing Rb phosphorylation that may also be considered an anti-proliferative senescence response. However, the loss of p53 activity can, in turn, bypass this senescence, thereby enhancing the malignant phenotype. Therefore, we suggest that SPN may be a novel tumor suppressor, reinforcing the role of PP1α as a tumor suppressor.

MATERIALS AND METHODS

Cell culture, retroviral vectors and gene transfer. Cells were generated and characterized following the same experimental procedure described in [59-60]. 3T3 protocol was conducted as previously described in [30]. Temperature shifts and cell proliferation analysis were performed as described in [61-62]. Proliferation Assays, were performed for MTT colorimetric read-out as previously described in [63].

Cells treatment with 3MC, Etoposide, doxorubicin and H_2O_2. Cells were seeded in six-well plates. Next day, cells at 50–75% confluence were treated with doxorubicin (0.4 or 0.8 µg/ml), Etoposide (100 mM), 3-Methylcholantrene (10µM) or with 100 µM H_2O_2, or UCN01 (50, 100 y 200nM) during the indicated times. After this period of time, cells were harvested and proteins analyzed as described in western blot analysis.

Growth in soft agar and foci formation. To measure the anchorage-independent growth and foci formation we follow a protocol described previously in [64]. BrDu incorporation. Was adapted from the protocol provided in the BrdU cell proliferation assay from Exalpha Biologicals, Inc. (Maynard, MA 01754, USA)

Cell cycle analysis was assessed using flow cytometry by propidium iodide (Sigma) staining as described in [65]. A total of 10,000 size gated cells were analyzed by FACSCalibur (BD Biosciences).

Design of shRNA against Spn. An shRNA against Spn was designed using the 'Ambion siRNA target finder' and the 'Qiagen siRNA design tool' to choose the appropriate hairpin oligonucleotides, which were then cloned in a pRetrosuper vector. An shRNA against PPP1CA was described in [23].

Generation, handling, and analysis of transgenic mice. All animal experiments were done under the experimental protocol approved by the Institutional Committee for Care and Use of Animals of the Spanish National Cancer Research Centre which complies with European legislation on the care and use of animals, NIH guidelines for the use of laboratory animals, and related codes of ethic practice. Spn KO generation and genotyping protocols are described in [18]. p53 KO [66] mice were obtained from Jackson laboratories.

Carcinogenic treatment with 3-Methylcholantrene (3MC). 3MC was dissolved in sesame oil at 10 mg/ml. cohorts of 20 mice between 3 and 5 months of age were intramuscularly injected with a dose of 1 mg of 3MC (100µl) or sesame oil only in the right back leg. Mice were examined weekly and sacrificed when the tumour grew to 1 cm in diameter.

Western Blot analysis. Total protein was extracted, processed and analyzed by western blot as described previously [67]. To detect the different proteins, membranes were hybridized with the following primary antibodies: anti-PP1α (protein phosphatase-1α) from Calbiochem; anti-Rb: G3-245 from BD PharMingen; anti-pRb phosphorylated: anti-pRb(Ser807/811) from Cell Signalling ; anti-α-tubulin: T9026 from Sigma. Anti-Spinophilin: AB5669 from Chemicon; anti-p53: p53 FL 393 (sc-6243) from Santa Cruz: anti- p21: C-19 (sc-397) from Santa Cruz. Anti- CycA; sc-751 from Santa Cruz. Anti-p16ink4a: (m-156) from Santa Cruz sc-1207. anti-p19ARF: ab80 from Abcam (104996). The membrane was then incubated with secondary antibody containing the horseradish peroxidase antimouse IgG (Promega, Germany) or antirabbit IgG (Calbiochem, San Diego CA), and developed with a detection system for chemiluminescence (Amersham Biosciences, UK).

Protein phosphatase assays. PP1 activity was determined using standard procedures, as described by the vendor (Anaspec) [66]. PP activity was assayed using pNPP as a substrate, which detects both PP1 and PP2A activities. To selectively quantify PP1 activity, we used 2.5 nM okadaic acid to selectively inhibit PP2A and 2.5 μM okadaic acid to inhibit PP1. We measured PP1 activity as the difference in activity measured at 2.5 nM and 2.5 μM okadaic acid. The cell pellet was homogenized in extraction buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 15 mM β-mercaptoethanol, 0.25 M sucrose, 0.3% Triton X-100, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) and centrifuged to produce a soluble supernatant. The PP activity in the cleared supernatant was determined by measuring the absorbance at 405 nM. The incubation for determining PP activity was 10 min, and each assay contained 5 μg of protein from the cell extracts, as determined using the Bio-Rad assay (Bio-Rad, Hercules, CA).With these conditions, the PP activity was linear.

Statistical analysis. All statistics were analyzed using the SPSS statistical package (version 13.0 for Windows). A *p* value less than 0.05 was considered statistically significant.

Immunostaining and confocal analysis for co-localization was performed as previously indicated in [68]. The nuclei were stained with Hoechst 33258, for 3 min at room temperature prior to mounting with mowiol (Calbiochem). Images were

collected by confocal laser microscopy (model TCS-SP2-AOBS, Leica, Germany). Antibodies used were: Anti-Spinophilin: AB5669 from Chemicon; anti-p19ARF: ab80 from Abcam (104996); anti-p14ARF ab80 from Abcam (104996).

Figure Legends

Figure 1. **Loss of *Spn* affects Rb phosphorylation by decreasing the PPP1CA level**. **A)** Spn-null MEFs express a lower level of PPP1CA. MEFs from Spn KO mice and WT littermates were grown, and protein was extracted from the cells after the same number of population doublings. PPP1CA expression was examined using western blot. Three independent Spn-null MEF clones and three WT clones out of twenty that were analyzed for each genotype are shown. **B)** Quantification of PPP1CA in Spn-null MEFs. The PPP1CA level in 20 Spn-null clones and 20 WT clones was quantified and normalized to the tubulin level in each clone. The PPP1CA levels in the Spn-null clones were compared to the PPP1CA levels in the WT clones using an ANOVA. **C)** PPP1CA expression in Spn-null MEFs is not increased following expression of oncogenic Ras. MEFs from Spn KO mice or WT littermates were grown and at passage two, infected with a retrovirus expressing Hras-val12 (ras^{v12}) or the vector alone (V). After selection for 4 days, total protein was extracted, and PPP1CA expression was examined using western blot. The experiment was performed three independent times, and similar results were obtained. **D)** Quantification of the results obtained in the experiments performed in C. Left panel: average PPP1CA expression normalized to tubulin expression. Right panel: SPN level in WT MEFs. **E)** PP1 activity in Spn KO and WT MEFs. Exponentially growing MEFs from Spn KO mice and WT littermates at passage three were serum starved, and the PP1 phosphatase activity was measured. **F)** The data shown are the average from 20 Spn-KO clones and 20 WT clones. **G)** Inhibition of proteasome-mediated degradation increases the PPP1CA level in Spn-null cells. MEFs from Spn KO mice and WT littermates were grown and treated for 24 hrs with 10 nM MG132. Protein was extracted from cells at the same population doublings in all clones. PPP1CA expression was examined using western blot. Two independent Spn-null MEF

clones and WT clones out of the ten clones analyzed for each genotype are shown. **H)** The data show the average measurements of ten Spn KO clones and ten WT clones.

Figure 2. Lack of SPN increases Rb phosphorylation. **A)** Increased level of phosporylated Rb in Spn-null MEFs. Presenescent WT (+/+) and Spn-null (-/-) MEFs at passage two were grown in the presence of 10% FBS (+FBS). The cells were then serum starved for 24 hrs (0 h), after which 10% serum was added to the medium. Protein was extracted at the indicated time points. Total protein was resolved using PAGE, and Rb phosphorylated at Ser807/811 (pRb) was examined using western blot. The experiment was performed more than ten independent times, with similar results. **B)** Quantification of the experiment shown in A. The phosphorylated Rb level was normalized to the tubulin level in the same gel. Each point represents the average of five experiments, and the bars indicate the SD. **C)** DNA synthesis occurs more rapidly in Spn-null MEFs. Presenescent WT (+/+) and Spn-null (-/-) MEFs at passage two were grown in the presence of 10% FBS (+FBS). The cells were then serum starved for 24 hrs (0 h), after which 10% serum was added to the medium, together with BrdU. The cells were harvested at different time points, and BrdU incorporation was quantified as indicated in the Materials and Methods. The experiment was performed three independent times, with similar results. **D)** Cyclin A is increased and activated early in Spn-null MEFs. Presenescent WT (+/+) and Spn-null (-/-) MEFs at passage two were grown in the presence of 10% FBS (+FBS). The cells were then serum starved for 24 hrs (0 h), after which 10% serum was added to the medium. Protein was extracted at different time points following the addition of serum. Total protein was resolved using PAGE, and cyclin A expression was examined using western blot. **E)** Quantification of the results obtained in the experiments performed in D. The average cyclin A level was normalized to tubulin expression in three independent experiments; the bars indicate the SD.

Figure 3. The absence of *Spn* protects from p16 loss by promoting *p53* mutation during MEF immortalization. We generated multiple clones from WT (+/+), heterozygous (+/-), and Spn-null (-/-) MEFs and immortalized these cultures using standard 3T3 protocols. After immortalization at passage 11 and 12, total protein was extracted from each independent clone and resolved using PAGE. The expression of p53, p16, p19ARF, and tubulin was examined using western blot. The upper panels

show the results for WT (Spn (+/+)), heterozygous (Spn (+/-)), or Spn-null (Spn (-/-)) MEFs. The lower panels present the statistical analysis of the correlation between *p53* mutation, loss of p16 or p19ARF, and immortalization in the different clones. While we detected *p53* mutations based on the stabilization of p53, all mutations were confirmed by sequencing *p53* mRNA.

Figure 4. Loss of *Spn* increases p53 activity. A) p53-null MEFs expressing the thermosensitive mutant p53val145 growing at 39°C were infected with a retrovirus expressing two independent Spn-specific shRNAs (Sh1 and Sh2) and selected. The cells were seeded in triplicate and cultured at 32°C. After 2 weeks, the colony number in each culture was quantified. The upper panel shows the reduction of the SPN level by the shRNAs. The bottom panel presents the number of colonies growing at 32°C. **B)** Presenescent WT (+/+) and Spn-null (-/-) MEFs at passage two were grown in the presence of 10% FBS. Etoposide (Eto) was then added to the medium for 24 hrs (24 h), and total protein was extracted. The expression of p53, p21, SPN, and tubulin was examined using western blot. The bar graphs present the quantification of the p53 and p21 levels. The experiment was performed three independent times, with similar results. **C)** Presenescent WT (+/+) and Spn-null (-/-) MEFs at passage two were grown in the presence of 10% FBS. 3-MC was then added, and total protein was extracted at different time points. The expression of p21 and tubulin was examined using western blot. The bar graphs show the quantification of the p21 level normalized to tubulin expression. The experiment was performed three independent times, with similar results. **D)** and **E)** Cohorts of 13–15 mice were intramuscularly injected with 3-MC, and the appearance of tumors and survival of the mice were monitored. The graphs show the survival of the different cohorts: WT (Spn (+/+)), heterozygous (Spn (+/-)) or Spn-null (Spn (-/-)) mice on a WT p53 background (D) or heterozygous p53 (p53 (+/-)) background (E).

Figure 5. PPP1CA and ARF mediate SPN loss-dpendent p53 increase. A) Constitutive expression of PPP1CA inhibits the enhanced p53 activation resulting from the loss of *Spn*. Spn-null (-/-) and WT (+/+) MEFs were infected with a retrovirus expressing full-length PPP1CA (PPP1CA) or the vector alone (V). After selection, the cells were treated with 3-MC for 6 hrs or etoposide for 2 hrs, and the p53 levels were examined using western blot. The upper panel show the p53 and tubulin levels. The

bottom panel shows the quantification of one representative experiment out of three independent experiments. **B)** The loss of *Spn* increases p19ARF expression. Spn null (-/-) and WT (+/+) MEFs were infected with a retrovirus expressing Hras-val12 (RasV12), the p53 mutant 175H (p53DN), or the vector alone (V). After selection for 4 days, the cells were serum starved, and the p53 level was examined using western blot. The upper panel presents the p19ARF and tubulin levels, while the lower panel presents a quantification of one representative experiment out of three independent experiments. **C)** The p19ARF-specific shRNA decreases p19ARF expression in Spn-null MEFs. MEFs were transfected with pRetrosuper encoding a p19ARF-specific shRNA and selected for 7 days. After selection, the cells were serum starved, and the p19ARF protein levels were analyzed using western blot. **D)** The loss of ARF abolishes p53-enhanced induction following RasV12 expression. Wild-type (Spn (+/+)) or Spn-null (Spn (-/-)) MEFs expressing the p19ARF-specific shRNA or vector alone were infected with a retrovirus expressing oncogenic Ras or vector alone. After selection, the cells were serum starved, and the p53 and tubulin levels were examined. E) Immunofluoprescence demonstrating the lack of SPN and ARF colocalization.

Figure 6. The absence of *Spn* on a p53-mutant background increases the tumorigenic potential of cells. A) p53-null MEFs expressing the thermosensitive mutant p53val145 growing at 39ºC were infected with a retrovirus vector carrying two independent Spn-specific shRNAs (Sh1 and Sh2) and selected. The cells were seeded in triplicate and grown at the permissive temperature (39ºC). After 1 week, the number of colonies in each culture was quantified. The graph shows the average colony number in three independent experiments. **B)** Immortalized WT (Spn +/+) and Spn-null (Spn-/-) MEF clones carrying mutations in *p53* were seeded in triplicate at a low density and grown at 37ºC. The cells were then serum starved, and the cell number was determined. The experiment was performed three independent times, with similar results. **C)** Different immortalized MEF clones, with the indicated genetic alterations, were grown in the presence of 10% FBS. The cells were serum starved for 24 hrs (-FBS), after which 10% serum was added to the medium (+FBS, 0 h). The cell number was determined at the indicated time points. **D)** Focus formation assay following expression of the *Ras* or *Ras+E1A* oncogenes in immortalized MEF clones with the indicated genotypes. **E**: The overexpression of SPN reduces the growth of tumor cells.

REFERENCES

1. Querzoli P, Albonico G, di Iasio MG, Ferretti S, Rinaldi R, Cariello A, et al. Biophenotypes and survival of BRCA1 and TP53 deleted breast cancer in young women. Breast Cancer Res Treat 2001;66:135-42.
2. Caduff RF, Svoboda-Newman SM, Ferguson AW, Frank TS. Comparison of alterations of chromosome 17 in carcinoma of the ovary and of the breast. Virchows Arch 1999;434:517-22.
3. Maitra A, Tavassoli FA, Albores-Saavedra J, Behrens C, Wistuba, II, Bryant D, et al. Molecular abnormalities associated with secretory carcinomas of the breast. Hum Pathol 1999;30:1435-40.
4. Abujiang P, Mori TJ, Takahashi T, Tanaka F, Kasyu I, Hitomi S, et al. Loss of heterozygosity (LOH) at 17q and 14q in human lung cancers. Oncogene 1998;17:3029-33.
5. Porter DE, Steel CM, Cohen BB, Wallace MR, Carothers A, Chetty U, et al. Genetic linkage analysis applied to unaffected women from families with breast cancer can discriminate high- from low-risk individuals. Br J Surg 1993;80:1381-5.
6. Porter DE, Cohen BB, Wallace MR, Carothers A, Steel CM. Linkage mapping in familial breast cancer: improved localisation of a susceptibility locus on chromosome 17q12-21. Int J Cancer 1993;53:188-98.
7. Porter DE, Cohen BB, Wallace MR, Smyth E, Chetty U, Dixon JM, et al. Breast cancer incidence, penetrance and survival in probable carriers of BRCA1 gene mutation in families linked to BRCA1 on chromosome 17q12-21. Br J Surg 1994;81:1512-5.
8. Cohen BB, Porter DE, Wallace MR, Carothers A, Steel CM. Linkage of a major breast cancer gene to chromosome 17q12-21: results from 15 Edinburgh families. Am J Hum Genet 1993;52:723-9.
9. Smith SA, Easton DF, Ford D, Peto J, Anderson K, Averill D, et al. Genetic heterogeneity and localization of a familial breast-ovarian cancer gene on chromosome 17q12-q21. Am J Hum Genet 1993;52:767-76.
10. Easton DF, Bishop DT, Ford D, Crockford GP. Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. Am J Hum Genet 1993;52:678-701.
11. Tseng SL, Yu IC, Yue CT, Chang SF, Chang TM, Wu CW, et al. Allelic loss at BRCA1, BRCA2, and adjacent loci in relation to TP53 abnormality in breast cancer. Genes Chromosomes Cancer 1997;20:377-82.
12. Allen PB, Ouimet CC, Greengard P. Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. Proc Natl Acad Sci U S A 1997;94:9956-61.
13. Satoh A, Nakanishi H, Obaishi H, Wada M, Takahashi K, Satoh K, et al. Neurabin-II/spinophilin. An actin filament-binding protein with one pdz domain localized at cadherin-based cell-cell adhesion sites. J Biol Chem 1998;273:3470-5.
14. Sarrouilhe D, di Tommaso A, Metaye T, Ladeveze V. Spinophilin: from partners to functions. Biochimie 2006;88:1099-113.
15. Vivo M, Calogero RA, Sansone F, Calabro V, Parisi T, Borrelli L, et al. The human tumor suppressor arf interacts with spinophilin/neurabin II, a type 1 protein-phosphatase-binding protein. J Biol Chem 2001;276:14161-9.
16. Hsieh-Wilson LC, Allen PB, Watanabe T, Nairn AC, Greengard P. Characterization of the neuronal targeting protein spinophilin and its interactions with protein phosphatase-1. Biochemistry 1999;38:4365-73.

17. Ouimet CC, Katona I, Allen P, Freund TF, Greengard P. Cellular and subcellular distribution of spinophilin, a PP1 regulatory protein that bundles F-actin in dendritic spines. J Comp Neurol 2004;479:374-88.
18. Feng J, Yan Z, Ferreira A, Tomizawa K, Liauw JA, Zhuo M, et al. Spinophilin regulates the formation and function of dendritic spines. Proc Natl Acad Sci U S A 2000;97:9287-92.
19. Tsukada M, Prokscha A, Eichele G. Neurabin II mediates doublecortin-dephosphorylation on actin filaments. Biochem Biophys Res Commun 2006;343:839-47.
20. Bielas SL, Serneo FF, Chechlacz M, Deerinck TJ, Perkins GA, Allen PB, et al. Spinophilin facilitates dephosphorylation of doublecortin by PP1 to mediate microtubule bundling at the axonal wrist. Cell 2007;129:579-91.
21. Berndt N. Protein dephosphorylation and the intracellular control of the cell number. Front Biosci 1999;4:D22-42.
22. Liu CW, Wang RH, Dohadwala M, Schonthal AH, Villa-Moruzzi E, Berndt N. Inhibitory phosphorylation of PP1alpha catalytic subunit during the G(1)/S transition. J Biol Chem 1999;274:29470-5.
23. Castro ME, Ferrer I, Cascon A, Guijarro MV, Lleonart M, Cajal SR, et al. PPP1CA contributes to the senescence program induced by oncogenic Ras. Carcinogenesis 2008;29:491-9.
24. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer 2009;9:153-66.
25. Sherr CJ. Principles of tumor suppression. Cell 2004;116:235-46.
26. Noonan EJ, Place RF, Basak S, Pookot D, Li LC. miR-449a causes Rb-dependent cell cycle arrest and senescence in prostate cancer cells. Oncotarget 2010;1:349-58.
27. Ayllon V, Martinez AC, Garcia A, Cayla X, Rebollo A. Protein phosphatase 1alpha is a Ras-activated Bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. Embo J 2000;19:2237-46.
28. Strack S, Barban MA, Wadzinski BE, Colbran RJ. Differential inactivation of postsynaptic density-associated and soluble Ca2+/calmodulin-dependent protein kinase II by protein phosphatases 1 and 2A. J Neurochem 1997;68:2119-28.
29. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. Cancer Cell 2002;2:103-12.
30. Carnero A, Hudson JD, Price CM, Beach DH. p16INK4A and p19ARF act in overlapping pathways in cellular immortalization. Nat Cell Biol 2000;2:148-55.
31. Chao SK, Horwitz SB, McDaid HM. Insights into 4E-BP1 and p53 mediated regulation of accelerated cell senescence. Oncotarget 2011;2:89-98.
32. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 1993;366:704-7.
33. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997;88:593-602.
34. Quelle DE, Cheng M, Ashmun RA, Sherr CJ. Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF. Proc Natl Acad Sci U S A 1997;94:669-73.
35. Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, et al. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell 1997;91:649-59.
36. Ferrer I PS, Cañamero M, Cecilia Y, Blanco-Aparicio C and Carnero A. Spinophilin loss contributes to tumorigenesis in vivo Cell Cycle 2011;in press.

37. Bates S, Phillips AC, Clark PA, Stott F, Peters G, Ludwig RL, et al. p14ARF links the tumour suppressors RB and p53. Nature 1998;395:124-5.
38. Palmero I, Pantoja C, Serrano M. p19ARF links the tumour suppressor p53 to Ras. Nature 1998;395:125-6.
39. Laurie NA, Donovan SL, Shih CS, Zhang J, Mills N, Fuller C, et al. Inactivation of the p53 pathway in retinoblastoma. Nature 2006;444:61-6.
40. Gallagher S, Kefford RF, Rizos H. Enforced expression of p14ARF induces p53-dependent cell cycle arrest but not apoptosis. Cell Cycle 2005;4:465-72.
41. Efeyan A, Garcia-Cao I, Herranz D, Velasco-Miguel S, Serrano M. Tumour biology: Policing of oncogene activity by p53. Nature 2006;443:159.
42. Molina-Pinelo S, Ferrer, I., Blanco-Aparicio, C., Peregrino, S., Pastor, M.D., Vega, J.A., Suarez, R., Vergel, M., Marin, J.J., Hernandez-Losa, J., Ramon y Cajal, S., Paz-Ares, L. and Carnero.A. Downregulation of spinophilin in lung tumors contributes to tumorigenesis. J of Pathol 2011;In press.
43. Shmueli A, Gdalyahu A, Sapoznik S, Sapir T, Tsukada M, Reiner O. Site-specific dephosphorylation of doublecortin (DCX) by protein phosphatase 1 (PP1). Mol Cell Neurosci 2006;32:15-26.
44. Santra M, Liu XS, Santra S, Zhang J, Zhang RL, Zhang ZG, et al. Ectopic expression of doublecortin protects adult rat progenitor cells and human glioma cells from severe oxygen and glucose deprivation. Neuroscience 2006;142:739-52.
45. Santra M, Zhang X, Santra S, Jiang F, Chopp M. Ectopic doublecortin gene expression suppresses the malignant phenotype in glioblastoma cells. Cancer Res 2006;66:11726-35.
46. Santra M, Santra S, Roberts C, Zhang RL, Chopp M. Doublecortin induces mitotic microtubule catastrophe and inhibits glioma cell invasion. J Neurochem 2009;108:231-45.
47. Terry-Lorenzo RT, Carmody LC, Voltz JW, Connor JH, Li S, Smith FD, et al. The neuronal actin-binding proteins, neurabin I and neurabin II, recruit specific isoforms of protein phosphatase-1 catalytic subunits. J Biol Chem 2002;277:27716-24.
48. MacMillan LB, Bass MA, Cheng N, Howard EF, Tamura M, Strack S, et al. Brain actin-associated protein phosphatase 1 holoenzymes containing spinophilin, neurabin, and selected catalytic subunit isoforms. J Biol Chem 1999;274:35845-54.
49. Ragusa MJ, Dancheck B, Critton DA, Nairn AC, Page R, Peti W. Spinophilin directs protein phosphatase 1 specificity by blocking substrate binding sites. Nat Struct Mol Biol 2010;17:459-64.
50. Rubin E, Tamrakar S, Ludlow JW. Protein phosphatase type 1, the product of the retinoblastoma susceptibility gene, and cell cycle control. Front Biosci 1998;3:D1209-19.
51. del Arroyo AG, El Messaoudi S, Clark PA, James M, Stott F, Bracken A, et al. E2F-dependent induction of p14ARF during cell cycle re-entry in human T cells. Cell Cycle 2007;6:2697-705.
52. Baker DJ, Jin F, van Deursen JM. The yin and yang of the Cdkn2a locus in senescence and aging. Cell Cycle 2008;7:2795-802.
53. Wells NJ, Fry AM, Guano F, Norbury C, Hickson ID. Cell cycle phase-specific phosphorylation of human topoisomerase II alpha. Evidence of a role for protein kinase C. J Biol Chem 1995;270:28357-63.
54. Kuntziger T, Landsverk HB, Collas P, Syljuasen RG. Protein phosphatase 1 regulators in DNA damage signaling. Cell Cycle 2011;10.

55. Lehmann BD, Brooks AM, Paine MS, Chappell WH, McCubrey JA, Terrian DM. Distinct roles for p107 and p130 in Rb-independent cellular senescence. Cell Cycle 2008;7:1262-8.
56. Barker HM, Jones TA, da Cruz e Silva EF, Spurr NK, Sheer D, Cohen PT. Localization of the gene encoding a type I protein phosphatase catalytic subunit to human chromosome band 11q13. Genomics 1990;7:159-66.
57. Sait SN, Dal Cin P, Sandberg AA. Recurrent involvement of 11q13 in acute nonlymphocytic leukemia. Cancer Genet Cytogenet 1987;26:351-4.
58. Coignet LJ, Schuuring E, Kibbelaar RE, Raap TK, Kleiverda KK, Bertheas MF, et al. Detection of 11q13 rearrangements in hematologic neoplasias by double-color fluorescence in situ hybridization. Blood 1996;87:1512-9.
59. Carnero A, Hudson JD, Hannon GJ, Beach DH. Loss-of-function genetics in mammalian cells: the p53 tumor suppressor model. Nucleic Acids Res 2000;28:2234-41.
60. Ruiz L, Traskine M, Ferrer I, Castro E, Leal JF, Kaufman M, et al. Characterization of the p53 response to oncogene-induced senescence. PLoS ONE 2008;3:e3230.
61. Castro ME, del Valle Guijarro M, Moneo V, Carnero A. Cellular senescence induced by p53-ras cooperation is independent of p21waf1 in murine embryo fibroblasts. J Cell Biochem 2004;92:514-24.
62. Castro ME, Leal JF, Lleonart ME, Ramon YCS, Carnero A. Loss-of-function genetic screening identifies a cluster of ribosomal proteins regulating p53 function. Carcinogenesis 2008;29:1343-50.
63. Moneo V, Serelde BG, Leal JF, Blanco-Aparicio C, Diaz-Uriarte R, Aracil M, et al. Levels of p27(kip1) determine Aplidin sensitivity. Mol Cancer Ther 2007;6:1310-6.
64. Guijarro MV, Leal JF, Blanco-Aparicio C, Alonso S, Fominaya J, Lleonart M, et al. MAP17 enhances the malignant behavior of tumor cells through ROS increase. Carcinogenesis 2007;28:2096-104.
65. Link W, Oyarzabal J, Serelde BG, Albarran MI, Rabal O, Cebria A, et al. Chemical interrogation of FOXO3a nuclear translocation identifies potent and selective inhibitors of phosphoinositide 3-kinases. J Biol Chem 2009;284:28392-400.
66. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr., Butel JS, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 1992;356:215-21.
67. Guijarro MV, Link W, Rosado A, Leal JF, Carnero A. MAP17 inhibits Myc-induced apoptosis through PI3K/AKT pathway activation. Carcinogenesis 2007;28:2443-50.
68. Blanco-Aparicio C, Pequeno B, Moneo V, Romero L, Leal JF, Velasco J, et al. Inhibition of phosphatidylinositol-3-kinase synergizes with gemcitabine in low-passage tumor cell lines correlating with Bax translocation to the mitochondria. Anticancer Drugs 2005;16:977-87.