



Spleen tyrosine kinase functions as a tumor suppressor in melanoma cells by inducing senescence-like growth arrest.

Olivier Bailet, Nina Fenouille, Patricia Abbe, Guillaume Robert, Stéphane Rocchi, Nadège Gonthier, Christophe Denoyelle, Michel Ticchioni, Jean-Paul Ortonne, Robert Ballotti, et al.

► To cite this version:

Olivier Bailet, Nina Fenouille, Patricia Abbe, Guillaume Robert, Stéphane Rocchi, et al.. Spleen tyrosine kinase functions as a tumor suppressor in melanoma cells by inducing senescence-like growth arrest.: Syk activates p53 signaling pathway. *Cancer Research*, 2009, 69 (7), pp.2748-56. 10.1158/0008-5472.CAN-08-2690 . inserm-00371643

HAL Id: inserm-00371643

<https://inserm.hal.science/inserm-00371643>

Submitted on 8 Mar 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Spleen tyrosine kinase functions as a tumor suppressor in melanoma cells by inducing senescence-like growth arrest

Olivier Baillet¹, Nina Fenouille¹, Patricia Abbe¹, Guillaume Robert¹, Stéphane Rocchi¹, Nadège Gonthier², Christophe Denoyelle¹, Michel Ticchioni², Jean-Paul Ortonne^{1,3}, Robert Ballotti¹, Marcel Deckert^{2,4}, Sophie Tartare-Deckert^{1,3,*}

¹ C3M, Centre méditerranéen de médecine moléculaire INSERM : U895, Université de Nice Sophia-Antipolis, FR

² Régulations des réactions immunitaires et inflammatoires INSERM : U576, IFR50, Université de Nice Sophia-Antipolis, Hôpital de L'Archet 151, Rte de St Antoine de Ginestiere 06202 NICE CEDEX 3, FR

³ Service de Dermatologie CHU Nice, Hôpital l'Archet, FR

⁴ Service d'Hématologie CHU Nice, Hôpital l'Archet, FR

* Correspondence should be addressed to: Sophie Tartare-Deckert <tartare@unice.fr>

Abstract

Loss of tumor suppressive pathways that control cellular senescence is a crucial step in malignant transformation. Spleen tyrosine kinase (Syk) is a cytoplasmic tyrosine kinase that has been recently implicated in tumor suppression of melanoma, a deadly skin cancer deriving from pigments-producing melanocytes. However, the mechanism by which Syk suppresses melanoma growth remains unclear. Here we report that re-expression of Syk in melanoma cells induces a p53-dependent expression of the cyclin-dependent kinase (cdk) inhibitor p21 and a senescence program. We first observed that Syk expression is lost in a subset of melanoma cell lines, primarily by DNA methylation-mediated gene silencing and restored after treatment with the demethylating agent 5-Aza-2-deoxycytidine. We analyzed the significance of epigenetic inactivation of Syk and found that reintroduction of Syk in melanoma cells dramatically reduces clonogenic survival and three-dimensional tumor spheroid growth and invasion. Remarkably, melanoma cells re-expressing Syk display hallmarks of senescent cells, including reduction of proliferative activity and DNA synthesis, large and flattened morphology, senescence-associated β -galactosidase activity and heterochromatic foci. This phenotype is accompanied with hypophosphorylated retinoblastoma protein (Rb) and accumulation of p21, which depends on functional p53. Our results highlight a new role for Syk tyrosine kinase in regulating cellular senescence and identify Syk-mediated senescence as a novel tumor suppressor pathway whose inactivation may contribute to melanoma tumorigenicity.

MESH Keywords Cell Aging ; physiology ; Cell Growth Processes ; physiology ; Chemotaxis ; Cyclin-Dependent Kinase Inhibitor p21 ; biosynthesis ; genetics ; DNA Methylation ; Humans ; Intracellular Signaling Peptides and Proteins ; genetics ; metabolism ; physiology ; Melanoma ; enzymology ; genetics ; pathology ; Neoplastic Stem Cells ; pathology ; Promoter Regions, Genetic ; Protein-Tyrosine Kinases ; biosynthesis ; genetics ; metabolism ; physiology ; RNA, Small Interfering ; genetics ; Signal Transduction ; Spheroids, Cellular ; Transfection ; Tumor Suppressor Protein p53 ; metabolism ; Up-Regulation ; src Homology Domains

Author Keywords Melanoma ; tumor suppressor ; senescence

INTRODUCTION

Cutaneous melanoma is the leading cause of skin cancer-related mortality in Western societies owing to its high capability of invasion and rapid metastasis to other organs (1). The first step toward transition to melanoma is uncontrolled proliferation of epidermal melanocytes and dysplasia that may arise within a preexisting benign lesion (nevus) or more frequently directly from a new site. Subsequently, primary melanoma progresses through the radial growth phase (RGP) characterized by horizontal growth of transformed cells within the epidermis and the vertical growth phase (VGP), which is associated with depth invasion of cells into dermis and acquisition of metastatic potential (2). Melanoma formation and progression is accompanied by activating mutations in B-RAF, N-RAS and KIT genes as well as in CDK4 gene and loss of tumor suppressor genes such as CDKN2A, PTEN and E-cadherin (2, 3). Oncogenic signaling and failure of tumor suppressor mechanisms are believed to contribute to the molecular pathogenesis of melanoma.

Cellular senescence represents a powerful tumor-suppressive process that constrains malignant transformation, as it controls excessive proliferation driven by oncogenic mutations (4–7). B-RAF mutations are the most prevalent oncogenic mutations in human melanocytic skin lesions (8). B-RAF^{V600E} mutation was found in over two-thirds of human melanoma but also in a high proportion of benign nevi in which melanocytes were thought to be senescent. Michaloglou et al. (9) demonstrated that B-RAF^{V600E} activates a senescence program that induces melanocyte growth arrest, and that senescence in nevus cells is triggered by B-RAF^{V600E} signaling. This work supports the model that nevi represent senescent clones of melanocytes and that senescence is a barrier to melanoma progression (10). In this context, senescence was shown to be dependent on p16^{INK4a} expression (11). The B-RAF^{V600E} study, however, has suggested that besides p16^{INK4a} another melanoma suppressor(s) contribute to protection against oncogenic B-RAF signaling (9). In this regard, the secreted protein IGFBP7 has been recently proposed to contribute to B-RAF^{V600E}-mediated cell senescence (12).

Spleen tyrosine kinase (Syk) is a nonreceptor tyrosine kinase that is widely expressed in hematopoietic cells. It contains tandem N-terminal Src homology 2 (SH2) domains, multiple tyrosine phosphorylation sites and a C-terminal tyrosine kinase domain. The SH2 domains bind phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) and hence couple activated immunoreceptors to multiple downstream signaling pathways. Syk is essential for lymphocyte development and function, and signal transduction via a variety of membrane receptors in non-lymphoid cells such as mast cells or platelets (13). It was believed for years that Syk function was solely linked with hematopoietic cell signaling. However, more recent studies have indicated a ubiquitous pattern of Syk gene expression. Syk is expressed in endothelial cells, fibroblasts, epithelial cells and neuronal cells, but its function in these cells is not yet completely understood (14).

Accumulating evidence suggests that Syk can function as a tumor suppressor, unlike other tyrosine kinases that generally promote growth-stimulating activity leading to tumorigenicity (15). The role of Syk as candidate tumor suppressor has been well documented in breast cancer. Loss or reduced expression of Syk in human breast cancers was associated with a higher degree of malignancy and poor prognosis (16–18). Consistent with a role for Syk inactivation in tumor progression, re-expression of Syk in invasive breast cancer cells was shown to inhibit tumor growth and reduce metastasis in mouse xenografts (16). The tumor suppressive activity of Syk in breast cancer cells has been associated with abnormal mitotic progression and cell death (18, 19). Epigenetic silencing through hypermethylation of critical CpG islands was proposed to be involved in loss of Syk in a significant fraction of breast tumors (20). More recently, a similar loss of Syk expression has been documented in melanoma cells. Importantly, reintroduction of Syk was shown to restrict tumor growth and metastasis in vivo (21, 22). However, the molecular events responsible for Syk tumor suppressor effects in melanoma cells have remained completely unknown.

Here we aimed to investigate the tumor suppressor function of Syk in melanoma cells. We present evidence that Syk exerts its antitumor activity through induction of a senescence-like growth arrest and activation of a p53-dependent pathway, suggesting that loss of Syk may contribute to the senescence bypass generally observed in malignant melanoma. Our findings identify a novel link between the tyrosine kinase Syk, p53 and the senescent program in cancer cells.

MATERIALS AND METHODS

Reagents and plasmids

The list of antibodies used is provided in supplementary methods. Pifithrin- α (PFT- α) was from Calbiochem. p53 small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology. Wild-type and kinase-defective (Syk-KD, K395R) porcine Syk cDNAs as well as SH2 domains-deleted Syk mutant (Syk- Δ SH2) were cloned into pEF6/V5 vector. These mutants have been described elsewhere (23). The SH2-inactive domains mutant (Syk-SH2D) was produced using a QuikChange site-directed mutagenesis kit supplied by Stratagene. Recombinant adenoviruses expressing wild-type and kinase-defective Syk were kindly provided by Dr Yamamura (Kobe University Japan) and have been described previously (24).

Cell culture, RNAi, and adenovirus infection

Human melanoma cell lines were provided by Drs M. Herlyn (Wistar Institute, Philadelphia, PA) and F. Jotereau (INSERM U601, Nantes, France). Cells were routinely maintained in DMEM supplemented with 7% FBS as described elsewhere (25). Transfection of duplex siRNAs (50nM) was carried out using Lipofectamine RNAiMAX (Invitrogen). Adenovirus infection was performed as described previously (26).

Analysis of cell proliferation, colony formation assays and three-dimensional spheroid growth

For BrdU incorporation and DNA-content analysis, subconfluent cultures were pulsed with 10 μ M BrdU for 4 hr. Cells were harvested and DNA stained with 7-amino-actinomycin D (7-AAD) and a APC-conjugated anti-BrdU antibody using the APC BrdU Flow Kit (BD Biosciences). Flow cytometric analyses were performed on a Becton Dickinson FACSCalibur machine and data were analysed using CellQuest software.

For colony formation assays, cells were transfected by using FuGENE 6 (Roche Diagnostics) with DNA mixtures containing 1 μ g of the relevant plasmids plus 0.1 μ g of pBabe-Puro vector. After 48 hr, cells were selected with 1 μ g/ml of puromycin and maintained for 12–15 days. Colonies were fixed with 3% paraformaldehyde and stained with 0.4% crystal violet. The number of colonies was scored quantitatively using Image J software.

Melanoma spheroids were generated using the liquid overlay technique as described (27). Briefly, 24-well culture plates were coated with 1.5% agarose prepared in sterile water. Cells from a single-cell suspension were added at 10,000 per well. The plates were gently swirled and incubated at 37°C in 5% CO₂ atmosphere until spheroid aggregates were formed.

Senescence-associated β -galactosidase (SA- β -gal) staining

Cells were washed in PBS and fixed with 3% formaldehyde for 15 min at room temperature. Staining for SA- β -gal activity was performed as described previously (28), utilizing the senescence β -galactosidase staining kit (Cell Signaling Technology). Stained cells were visualized by phase contrast microscopy, photographed and counted.

Immunoblotting and immunofluorescence

Whole cell lysates were prepared by lysing cells in radioimmunoprecipitation assay buffer supplemented with 1% NP40 and subjected to SDS-PAGE. Immunoblotting was accomplished according to standard procedures using enhanced chemiluminescence detection. For immunofluorescence, cells were fixed with 3% paraformaldehyde, and then incubated with primary and secondary antibodies as described elsewhere (25, 29).

Migration and invasion assays

Chemotaxis and Matrigel invasion assays were monitored using modified Boyden chambers as described (25, 29).

Statistical analysis

Unless indicated otherwise, experiments shown were representative of at least three independent experiments. Results were expressed as mean \pm SD. Where appropriate the Student's *t* test was done and *P* < 0.001 was considered statistically significant.

RESULTS

Syk expression and Syk promoter methylation status in melanocytes and melanoma cells

Initially, we examined expression, phosphorylation level and cellular localization of Syk in melanocytes. A 72 kDa band corresponding to Syk was readily detected by immunoblotting of lysates from different primary human melanocytes cultures (Supplementary Fig. S1). We also observed that Syk is phosphorylated on tyrosine 352, a site that has been proposed to bind signaling proteins (23, 30). Consistent with previous reports (21, 22), we observed that Syk is absent or nearly undetectable in most human primary and metastatic melanoma cells tested (Fig. S2). Methylation-specific PCR (MSP) studies showed that transcriptional silencing of Syk in Sbcl2, A375, 1205Lu and WM9 cells was associated with aberrant DNA methylation of the promoter region that we examined. In contrast, no methylation was observed for Syk-positive cell lines and melanocytes. However, we found that in Syk-negative WM793 cells, the promoter is unmethylated, suggesting that mechanism other than methylation of this region may be involved in SYK gene inactivation. Finally, we found that treatment with the demethylating drug, 5-azadC led to an increase of Syk mRNA levels whereas the histone deacetylase inhibitor TSA had no discernible effect on Syk mRNA expression (Fig. S2).

Syk decreases colony formation, tumor growth in 3D spheroids and chemotaxis

Having shown that Syk was inactivated in several melanoma cell lines, we examined its potential growth suppressor function in monolayer cultures and 3D spheroid model. We transfected a Syk-expression plasmid into melanoma cell lines and colony formation assays were done to evaluate long-term growth on plastic dishes (Fig. 1A). Reintroduction of Syk dramatically reduced colony formation efficiency by at least 70% in Syk-negative A375 and SKmel28 cells. The same experiment was also carried out in 501mel cells expressing endogenous Syk protein levels (Fig. 1A, 501mel lanes; see Fig. S2 for Syk blot of 501mel showing endogenous Syk level). In this setting as well, elevated expression of Syk led to inhibition of colony formation. Transient transfection showed that each of the cell lines were able to express comparable levels of exogenous V5 epitope-tagged Syk (Fig. 1A).

We also investigated the effect of Syk reintroduction on tumor spheroid growth using adenoviral delivery system. To exclude the possibility that this effect would be the result of overexpression of Syk, we determined the level of Syk re-expression in melanoma cells and we found that level of exogenous Syk in lysates made from infected A375 cells was comparable to endogenous level seen in melanocytes (see supplemental information Fig. S3). In all subsequent experiments, we infected cells with a MOI of 5, which is the dose of virus that induces physiological level of Syk expression. Culture of WM793 cells under nonadherent conditions resulted in generation of 3D multicellular tumor spheroids, which may be used as an experimental model to study the tumor biology context. We prepared 3D spheroids from cells infected with control or Syk-expressing adenoviruses. After 3 days, there was significant decrease in spheroids diameter when cells were infected with Ad-Syk compared with vector control. Expression of adenovirus-delivered Syk was confirmed by immunoblot analysis (Fig. 1B). Together, these observations indicate that Syk has growth-suppressing activity in standard monolayer cultures and 3D spheroids.

Finally, we analyzed the effect of Syk on melanoma cell migration and invasion towards serum using modified Boyden chambers assays. As seen Fig. 1C, re-expression of Syk markedly decreased A375 chemotactic migration and invasion. Because no further reduction was observed in the invasive capacity of cells, we can conclude that Syk primarily inhibits melanoma cell chemotaxis. Inhibition of tumor cell invasion into collagen I matrix by Syk was also evidenced in 3D cultures (Fig. S4).

Suppression of clonogenic survival by Syk is independent on functional SH2 domains but requires tyrosine kinase activity

Because A375 had complete methylation and silencing of Syk (Fig. S2) and was most sensitive to Syk-induced growth inhibition (Fig. 1A), we analyzed this cell line further. We conducted loss-of-function experiments, to define domain(s) of Syk that mediate growth suppression. We took advantage of a kinase-inactive mutant and generated a mutant that harbors inactivating mutations in the SH2 domains, as well as a mutant with a deletion of the two SH2 domains and interdomain A (Fig. 2A). Cells transfected with plasmids encoding these mutants or a wild-type control expressed similar levels of the corresponding proteins 3 days after transfection (Fig. 2B). Subsequently, these same transfections were subjected to colony-forming assay. As shown in Fig. 2C, expression of Syk SH2 mutants inhibited colony formation relative to vector control. Thus, Syk functions independently of its SH2 domains and interdomain A to suppress colony formation abilities of melanoma cells. However, mutation of the ATP-binding site did not significantly inhibit the ability to form colonies, indicating that enzymatic activity of Syk is required for its effect on growth inhibition.

Expression of Syk inhibits proliferation by modulating cell cycle progression

We undertook to determine how Syk reintroduction suppresses melanoma cell growth and survival. A375 were transduced with adenoviral vector expressing Syk and we analyzed ability of cells to proliferate by proliferation curves, cell cycle analysis and BrdU incorporation assays. Proliferation curves revealed that Syk re-expression markedly slowed growth of A375 cells (Fig. 3A). Levels of Syk expression in infected cells during the course of the experiment is shown Fig. 3B. We also examined cell cycle profiles of Syk-expressing and control cells 4 days after infection by flow cytometry (Fig. 3C). Restoration of Syk resulted in accumulation of cells in G0/G1 and decrease in S-phase cells compared with control cells, suggesting that Syk growth suppression results from G1 cell cycle arrest. In support of this, A375 cell population expressing Syk displayed a strong reduction in BrdU incorporation compared to vector-transduced cell population (Fig. 3D). Of note, cells transduced with Syk show no increase in sub-G1 phase and no caspase 3 activation, indicating that Syk expression did not result in cell death or apoptosis (Fig. 3A, C and data not shown).

Expression of Syk promotes senescence-like phenotypes

We observed that a major population of cells arrested by Syk displayed a flat enlarged morphology with increased granularity that is characteristic of senescent cells (Fig. 4A). This increase in cell size was also observed by flow cytometry-based assays where Syk-expressing cells showed elevated side scatter that is characteristic of senescent cells. Senescence is accompanied by a series of genetic and metabolic changes that include accumulation of SA β -gal. To confirm that the observed morphological changes were due to induction of senescence, we assessed SA β -gal activity 6 days post-infection. A blue staining indicative of acidic β -gal activity was observed in the growth-arrested A375 infected with Ad-Syk (Fig. 4B). Notably, about 40% of cells were β -gal positive in Syk-expressing populations, whereas less than 2% of A375 cells were positive in vector-infected cultures (Fig. 4C). Lastly, we looked for the presence of senescence-associated heterochromatin foci (SAHF), another feature of senescent cells. Immunofluorescence analysis for HP1 β , a marker of SAHF revealed punctuated regions of DNA corresponding to heterochromatic foci in Syk-expressing cells but not in control Syk negative cells (Fig. 4D). All together, these findings indicate that the cell-cycle arrest produced by Syk is mainly associated with induction of senescence-like phenotype.

Expression of Syk up-regulates the p53-signaling pathway

Next, we sought to understand the molecular pathway(s) involved in senescence-like growth arrest after Syk reintroduction. Immunoblot analysis was done to investigate expression and phosphorylation levels of specific proteins involved in cell cycle regulation. Increased levels of activating phosphorylation of p53 at Ser15 and induction of the cdk inhibitor p21 were observed in Syk-expressing A375 cells compared with controls (Fig. 5A). We also noticed that Syk expression resulted in modest, but consistent, increase in the levels of total p53. In addition, Syk-expressing cells harbored dephosphorylation of pRb and lost cyclin A expression. Consistent with changes in p53 regulation, immunofluorescence of individual cells showed nuclear p53 accumulation in Syk-positive cells (Fig. 5B). The nuclear accumulation of p53 was similar to the one observed after treatment with the radio-mimicking drug bleomycin. These data indicate that Syk expression results in up-regulation of p53/p21 and pRb pathways, which are known to mediate cell-cycle arrest and premature senescence.

To substantiate further these observations, we analyzed by quantitative RT-PCR expression level of more than 90 transcripts involved in p53-dependent cell cycle regulation and survival following Syk re-expression (see supplemental information and Table S1). 29 genes appeared to be reproducibly modulated by Syk and some of them have been validated at protein level (Fig. 5A and Fig. S5). Among the 14 genes up-regulated, 6 were known transcriptional targets of p53 (p21, Gadd45A, Reprimo, MDM2, BAX and SIVA). DNMT-1 has been recently implicated in p53-dependent repression of Survivin, cdc2/cdk1 and mitotic cdc25C (31). Accordingly, these 3 genes were found repressed in Syk expressing cells as well as other repression targets of p53 such as CHK1 and Cyclins B. Some relevant genes also down-regulated by Syk expression included Livin, E2F1, BRCA1, Cyclins A2 and E2 and c-Myc. Finally, we found that one of the genes deeply suppressed after Syk expression was Caspase 2, which is implicated in p53-mediated apoptosis (32). Inhibition of E2F1, Caspase 2 and survivin expression was confirmed at protein level (Fig. S5). Together, our results indicate that expression of Syk is linked

to altered expression of essential cell survival and cell cycle-regulatory genes that consequently results in G1 cell cycle arrest and inhibition of cell growth.

Inactivation of p53 prevents Syk-induced p21 expression

p21 is a well-characterized cdk inhibitor and a transcriptional target of p53 (33, 34). The p53/p21 axis plays a crucial role in mediating G1 arrest and senescence in normal and several tumor cells in response to multiple stress insults (4, 5). Our studies show that Syk-induced growth inhibition of melanoma cells is consistently associated with induction of this pathway. Therefore, we next investigated the requirement of p53 in Syk-induced p21 expression. To this end, A375 were incubated with the specific p53 inhibitor PFT- α (35). The level of p21 normally seen after Syk expression was significantly lower in PFT- α -treated cells than in control cells (Fig. 6A). Similarly, we observed that induction of p21 by bleomycin was partially suppressed in PFT- α -treated cells. To rule out the possible nonspecific effect of PFT- α , we used siRNA to inhibit p53 expression. Cells were transfected with p53-specific siRNA (or irrelevant control siRNA) and subsequently infected with adenovirus expressing Syk or empty vector as control. Fig. 6B shows that siRNA to p53 led to an efficient knockdown of p53 protein as well as p21 basal expression. This experiment also revealed that induction of p21 by Syk expression or bleomycin treatment was totally prevented in p53-silenced cells. These data indicate that p53 plays a functional role in mediating Syk-induced p21 expression. According to this, no induction of p21 was seen when Syk is expressed in MeWo cells, which have no functional p53 (Fig. 6C).

DISCUSSION

There is accumulating evidence that the Syk tyrosine kinase is a novel tumor suppressor in melanoma. However, its mechanism of action with respect to tumor suppression is not known. Here, we show that re-expression of Syk in melanoma cells at levels similar to that seen in melanocytes, results in suppression of chemotaxis, sustained cell cycle arrest, and senescence accompanied by molecular events associated with activation of the p53 tumor suppressor pathway. Our observations on the antiproliferative effect of Syk support the hypothesis that inactivation of SYK gene confers a specific growth advantage in neoplastic melanocyte cells and promotes migration of tumor cells. Together, our findings agree with recent observations showing that Syk expression in xenografted melanoma cells results in dramatic reduction of tumor growth and metastatic abilities (21, 22).

Consistent with a tumor suppressor function of Syk in melanoma, Syk levels are frequently low or undetectable in melanoma cells and primary tumors. The loss of expression occurs at transcriptional level, and, as a result of DNA hypermethylation (22). Our study identified a similar epigenetic regulation in the majority of melanoma cells analyzed. However, as shown in breast invasive tumors (18), our results suggested that mechanisms other than promoter methylation might account for loss of Syk expression in certain melanoma cells. Nevertheless, our results underscored a potential role of cytosine methylation in SYK gene inactivation in melanoma cells. It now remains to analyze the possible correlation between SYK methylation rate and clinicopathological parameters in melanoma tumors.

Inhibition of colony-forming activity by Syk provides a model system to study the mechanism by which Syk exerts its growth suppressive effect. We found that kinase activation of Syk and further downstream signaling are required for Syk-mediated inhibition of colony formation. In contrast, the SH2 domains of Syk are dispensable for the colony growth suppressive effect. This observation is consistent with the view that function of Syk in melanoma cells does not require the interaction of the SH2 domains with phosphorylated ITAM tyrosines on putative receptors or adaptor proteins. However, it is possible that Syk activity in melanoma depends on its subcellular localization. Reports have showed that repression of breast tumor cell growth by Syk is associated with its nuclear localization and transcriptional repressor function (36, 37). Also, loss of nuclear Syk expression was shown to be closely associated with the metastatic property of gastric cancer (38). Consistently, Zhou et al. (39) identified a shuttling sequence in Syk that accounts for its nucleocytoplasmic trafficking. It remains to be assessed whether such nuclear activity and localization of Syk occurs in melanoma cells and what are the structural element(s) of Syk involved in tumor suppression.

The growth suppression by Syk appears to be mediated through a G0/G1 cell cycle arrest and induction of senescence, and did not involve increased apoptosis or cell death. Interestingly, this activity seems specific to melanoma cells, as compared to breast carcinoma cells where expression of Syk triggered a non-apoptotic cell death through mitotic catastrophe (18, 19). This is consistent with the idea that Syk mediates senescence or death depending on the genetic background of the tumor. At this stage, the mechanism of tumor type-specific activity of Syk remains unclear.

p53 is a major regulator of cell-cycle arrest, senescence or apoptosis (40). Our findings provide the first evidence that tumor growth inhibition associated with Syk involves activation of p53 as reflected by phosphorylation of p53, nuclear accumulation and regulation of various p53-responsive genes. The net effect of these changes tips the balance in favor of proliferative arrest and protects cells from p53-dependent apoptosis. In support of this, expression of Caspase 2, a protease implicated in p53-mediated apoptosis (41, 32) was strongly suppressed following Syk re-expression. One of the major contributors of p53-directed cell cycle arrest and senescence is p21 (also known as senescent cell-derived inhibitor) (33, 41, 42), and we provide evidence for a functional requirement of p53 for p21 regulation by Syk. p21 functions by inhibiting cdk activity that results in hypophosphorylation of pRB and repression of E2F-targets

genes, which blocks S phase entry and mediates cell cycle arrest. According to this, expression of Syk is consistently associated with hypophosphorylation of pRB and inhibition of E2F-targets genes such as cyclin A and cyclin B. Importantly, we also found a dramatic loss of E2F1 gene expression that can also account for the proliferative arrest and senescence experienced by melanoma cells expressing Syk. A similar down-regulation of E2F1 has been recently reported in senescing melanoma cells treated by the anticancer agent diperpen ester (43).

At present, we do not know the mechanism(s) through which Syk promotes phosphorylation and activation of p53. Phosphorylation of p53 may involve ATM/ATR DNA damage signaling pathways. However, it seems unlikely, as we did not observe activation of this pathway in cells expressing Syk (unpublished observations). The stress-activated c-jun N-terminal kinase might be a candidate for the kinase that phosphorylates p53 (44). In this regard, it is interesting to note that Syk activates this pathway in melanoma cells (Fig. S6).

The results reported here might have important implications in our understanding of the physiological role of Syk in melanocytes. To date, the upstream signals involved in Syk activation and signaling pathways regulated by Syk in melanocytes are not known. However, because Syk modulates the p53 pathway in melanoma cells, we speculate that it may integrate p53-mediated stress responses in melanocytes. Accordingly, a link between Syk and oxidative stress signaling has been observed in B lymphocytes (39 , 45). Like apoptosis, senescence is a stress response that prevents neoplastic transformation. Based on our observations, we propose that loss of Syk during melanoma development can lead to altered stress-dependent responses resulting in uncontrolled proliferation of abnormal cells. Additional investigations using suppression of Syk in melanocytes will elucidate how inactivation of Syk contributes to melanoma pathogenesis.

To conclude, our results uncover a novel role for Syk in regulation of p53 and senescence program, and give a molecular explanation for its tumor suppressive function in melanoma. This provides an important insight for further understanding tumorigenic and invasive processes, particularly in the context that epigenetic inactivation of Syk is associated with numerous cancers.

Acknowledgements:

We thank H. Yamamura for Syk adenoviruses, M. Herlyn and F. Jotereau for melanoma cell lines. We also thank C. Gaggioli, G. Ponzio and R. Rottapel for helpful discussions.

Grant support : INSERM, ARC grant N° 3111 and INCa grant PL 2007. O. Bailet is a recipient of an INSERM-région Provence Alpes Côte d'Azur PhD fellowship in partnership with Galderma R&D (Sophia -Antipolis, France). S. Tartare-Deckert is a recipient of a Contrat d'Interface Clinique (CHU Nice).

References:

1. Chin L, Garraway LA, Fisher DE. Malignant melanoma: genetics and therapeutics in the genomic era. *Genes Dev*. 2006; 20: 2149 - 82
2. Miller AJ, Mihm MC Jr. Melanoma. *N Engl J Med*. 2006; 355: 51 - 65
3. Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. *J Clin Oncol*. 2006; 24: 4340 - 6
4. Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell*. 2005; 120: 513 - 22
5. Collado M, Serrano M. The senescent side of tumor suppression. *Cell Cycle*. 2005; 4: 1722 - 4
6. 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
7. Mooi WJ, Peeper DS. Oncogene-induced cell senescence--halting on the road to cancer. *N Engl J Med*. 2006; 355: 1037 - 46
8. Dhomen N, Marais R. New insight into BRAF mutations in cancer. *Curr Opin Genet Dev*. 2007; 17: 31 - 9
9. Michaloglou C, Vredeveld LC, Soengas MS. BRAF600-associated senescence-like cell cycle arrest of human naevi. *Nature*. 2005; 436: 720 - 4
10. Bennett DC. Human melanocyte senescence and melanoma susceptibility genes. *Oncogene*. 2003; 22: 3063 - 9
11. Gray-Schopfer VC, Cheong SC, Chong H. Cellular senescence in naevi and immortalisation in melanoma: a role for p16? *Br J Cancer*. 2006; 95: 496 - 505
12. Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR. Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell*. 2008; 132: 363 - 74
13. Turner M, Schweighoffer E, Colucci F, Di Santo JP, Tybulewicz VL. Tyrosine kinase SYK: essential functions for immunoreceptor signalling. *Immunol Today*. 2000; 21: 148 - 54
14. Yanagi S, Inatome R, Takano T, Yamamura H. Syk expression and novel function in a wide variety of tissues. *Biochem Biophys Res Commun*. 2001; 288: 495 - 8
15. Coopman PJ, Mueller SC. The Syk tyrosine kinase: A new negative regulator in tumor growth and progression. *Cancer Lett*. 2006;
16. Coopman PJ, Do MT, Barth M. The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. *Nature*. 2000; 406: 742 - 7
17. Toyama T, Iwase H, Yamashita H. Reduced expression of the Syk gene is correlated with poor prognosis in human breast cancer. *Cancer Lett*. 2003; 189: 97 - 102
18. Moroni M, Soldatenkov V, Zhang L. Progressive loss of Syk and abnormal proliferation in breast cancer cells. *Cancer Res*. 2004; 64: 7346 - 54
19. Zyss D, Montcourrier P, Vidal B. The Syk tyrosine kinase localizes to the centrosomes and negatively affects mitotic progression. *Cancer Res*. 2005; 65: 10872 - 80
20. Yuan Y, Mendez R, Sahin A, Dai JL. Hypermethylation leads to silencing of the SYK gene in human breast cancer. *Cancer Res*. 2001; 61: 5558 - 61
21. Hoeller C, Thallinger C, Pratscher B. The non-receptor-associated tyrosine kinase Syk is a regulator of metastatic behavior in human melanoma cells. *J Invest Dermatol*. 2005; 124: 1293 - 9
22. Muthusamy V, Duraisamy S, Bradbury CM. Epigenetic silencing of novel tumor suppressors in malignant melanoma. *Cancer Res*. 2006; 66: 11187 - 93
23. Deckert M, Tartare-Deckert S, Couture C, Mustelin T, Altman A. Functional and physical interactions of syk family kinases with the vav proto-oncogene product. *Immunity*. 1996; 5: 591 - 604
24. Inatome R, Yanagi S, Takano T, Yamamura H. A critical role for Syk in endothelial cell proliferation and migration. *Biochem Biophys Res Commun*. 2001; 286: 195 - 9
25. Robert G, Gaggioli C, Bailet O. SPARC represses E-cadherin and induces mesenchymal transition during melanoma development. *Cancer Res*. 2006; 66: 7516 - 23
26. Gaggioli C, Deckert M, Robert G. HGF induces fibronectin matrix synthesis in melanoma cells through MAP kinase-dependent signaling pathway and induction of Egr-1. *Oncogene*. 2005; 24: 1423 - 33

- 27 . Smalley KS , Haass NK , Brafford PA , Lioni M , Flaherty KT , Herlyn M . Multiple signaling pathways must be targeted to overcome drug resistance in cell lines derived from melanoma metastases . *Mol Cancer Ther* . 2006 ; 5 : 1136 - 44
- 28 . Dimri GP , Lee X , Basile G . A biomarker that identifies senescent human cells in culture and in aging skin in vivo . *Proc Natl Acad Sci U S A* . 1995 ; 92 : 9363 - 7
- 29 . Gaggioli C , Robert G , Bertolotto C . Tumor-derived fibronectin is involved in melanoma cell invasion and regulated by V600E B-Raf signaling pathway . *J Invest Dermatol* . 2007 ; 127 : 400 - 10
- 30 . Law CL , Chandran KA , Sidorenko SP , Clark EA . Phospholipase C-gamma1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk . *Mol Cell Biol* . 1996 ; 16 : 1305 - 15
- 31 . Esteve PO , Chin HG , Pradhan S . Human maintenance DNA (cytosine-5)-methyltransferase and p53 modulate expression of p53-repressed promoters . *Proc Natl Acad Sci U S A* . 2005 ; 102 : 1000 - 5
- 32 . Baptiste-Okoh N , Barsotti AM , Prives C . Caspase 2 is both required for p53-mediated apoptosis and downregulated by p53 in a p21-dependent manner . *Cell Cycle* . 2008 ; 7 : 1133 - 8
- 33 . el-Deiry WS , Tokino T , Velculescu VE . WAF1, a potential mediator of p53 tumor suppression . *Cell* . 1993 ; 75 : 817 - 25
- 34 . Sherr CJ , Roberts JM . CDK inhibitors: positive and negative regulators of G1-phase progression . *Genes Dev* . 1999 ; 13 : 1501 - 12
- 35 . Komarov PG , Komarova EA , Kondratov RV . A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy . *Science* . 1999 ; 285 : 1733 - 7
- 36 . Wang L , Devarajan E , He J , Reddy SP , Dai JL . Transcription repressor activity of spleen tyrosine kinase mediates breast tumor suppression . *Cancer Res* . 2005 ; 65 : 10289 - 97
- 37 . Wang L , Duke L , Zhang PS . Alternative splicing disrupts a nuclear localization signal in spleen tyrosine kinase that is required for invasion suppression in breast cancer . *Cancer Res* . 2003 ; 63 : 4724 - 30
- 38 . Nakashima H , Natsugoe S , Ishigami S . Clinical significance of nuclear expression of spleen tyrosine kinase (Syk) in gastric cancer . *Cancer Lett* . 2006 ; 236 : 89 - 94
- 39 . Zhou F , Hu J , Ma H , Harrison ML , Geahlen RL . Nucleocytoplasmic trafficking of the Syk protein tyrosine kinase . *Mol Cell Biol* . 2006 ; 26 : 3478 - 91
- 40 . Vousden KH , Lane DP . p53 in health and disease . *Nat Rev Mol Cell Biol* . 2007 ; 8 : 275 - 83
- 41 . Chang BD , Watanabe K , Broude EV . Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases . *Proc Natl Acad Sci U S A* . 2000 ; 97 : 4291 - 6
- 42 . Noda A , Ning Y , Venable SF , Pereira-Smith OM , Smith JR . Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen . *Exp Cell Res* . 1994 ; 211 : 90 - 8
- 43 . Cozzi SJ , Parsons PG , Ogbourne SM , Pedley J , Boyle GM . Induction of senescence in diterpene ester-treated melanoma cells via protein kinase C-dependent hyperactivation of the mitogen-activated protein kinase pathway . *Cancer Res* . 2006 ; 66 : 10083 - 91
- 44 . Fogarty MP , Downer EJ , Campbell V . A role for c-Jun N-terminal kinase 1 (JNK1), but not JNK2, in the beta-amyloid-mediated stabilization of protein p53 and induction of the apoptotic cascade in cultured cortical neurons . *Biochem J* . 2003 ; 371 : 789 - 98
- 45 . Takano T , Sada K , Yamamura H . Role of protein-tyrosine kinase syk in oxidative stress signaling in B cells . *Antioxid Redox Signal* . 2002 ; 4 : 533 - 41

Figure 1

Reintroduction of Syk in melanoma cells abrogates colony formation, growth in 3D culture and migration

(A) The indicated melanoma cell lines were transfected with a puromycin resistance vector and pEF-V5-wt-Syk expression vector or control empty vector (pEF). Stable puromycin-resistant colonies were stained and counted. Colony growth of cells transfected with control vector is normalized to 100% for each cell lines. Columns , mean of triplicate of a representative experiment; bars , SD. Representative plates are shown. A fraction of transfected cells was lysed and analyzed by immunoblotting for V5-tagged Syk expression. (B) Spheroids from WM793 cells infected with Syk-expressing (Ad-Syk) or empty vector adenoviruses were formed in liquid overlay cultures with agarose coat preventing cell adhesion to culture dishes. After 3 days, spheroids were viewed using a phase-contrast microscope and the diameter (μm) of at least 13 separate spheroids was determined. *, $P < 0.001$, Student's t test. Representative examples of spheroids are shown. Immunoblots show expression of Syk in spheroids transduced with control or with Syk-expressing adenoviruses. (C) Transwell migration and invasion assays of A375 infected with Syk-expressing or empty vector adenoviruses. The values are the mean \pm SD of three independent experiments. *, $P < 0.01$, Student's t test.

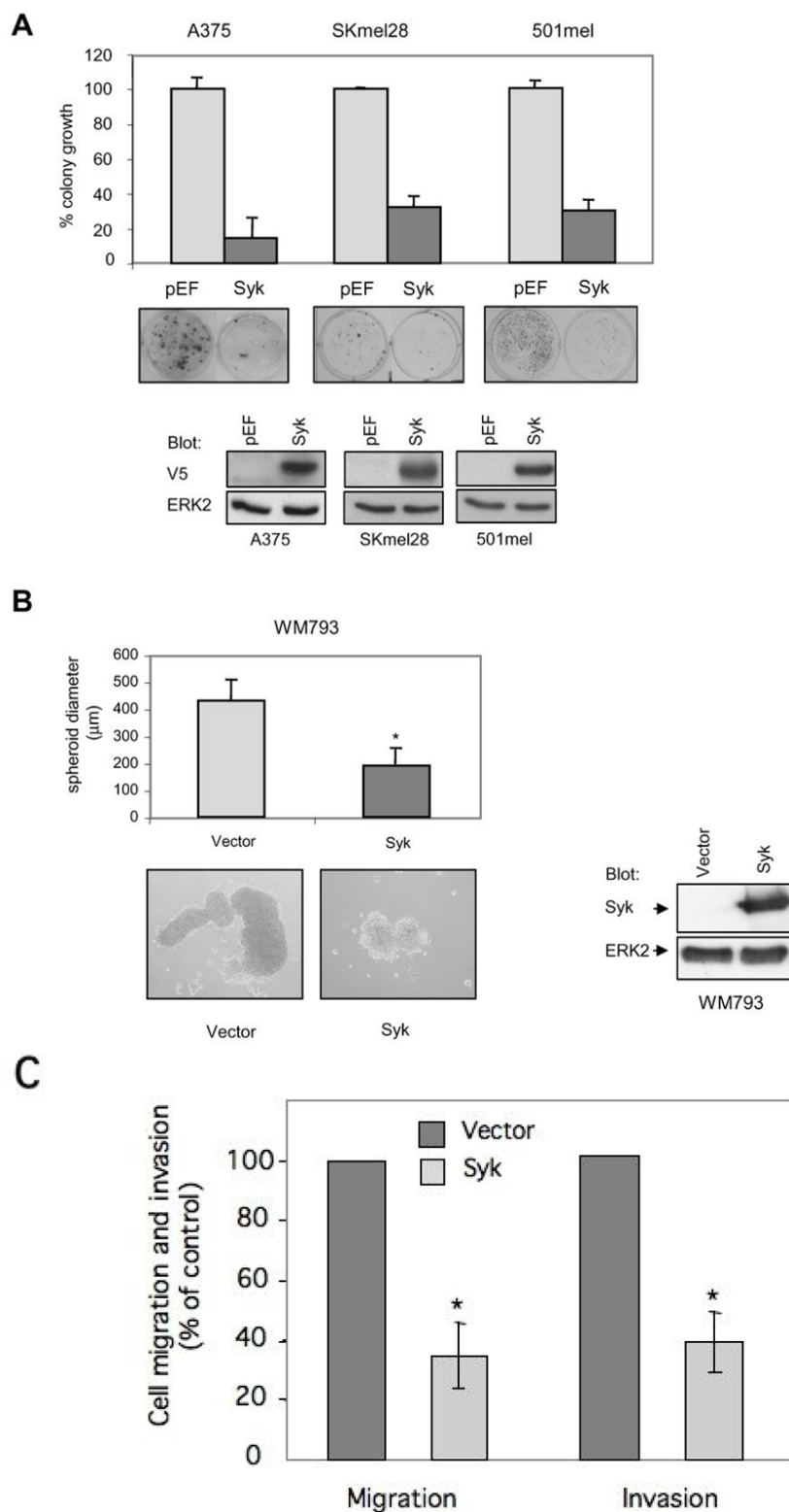


Figure 2

Syk suppresses colony growth independently of its two tandem SH2 domains

(A) Functional domains of Syk and schematic of the mutants. (B) Control immunoblot shows expression of the V5-tagged Syk wt and mutants in transiently transfected A375 cells. (C) Cells were transfected with empty vector (pEF), Syk wt, the SH2 deleted (Δ SH2), the SH2 mutated (SH2D) or the Kinase Dead (KD) Syk expression vector. Colony formation was analyzed as described in the legend of Fig. 1. Representative plates are shown. *, $P < 0.001$, Student's t tests.

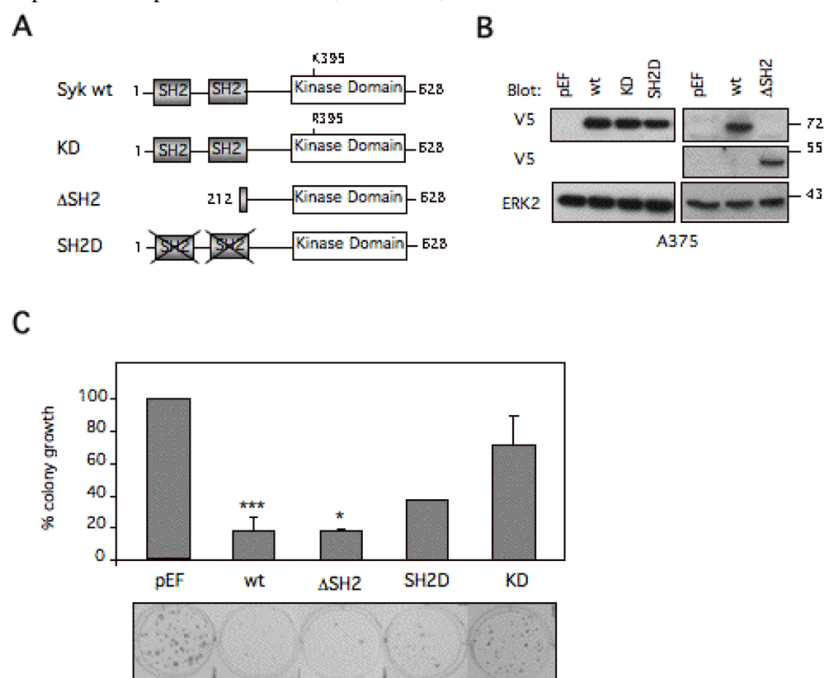


Figure 3

Syk reduces the growth of melanoma cells and induces a G0/G1 cell cycle arrest

(A) Proliferation curves of control and Syk-expressing cells. A375 cells were infected with control adenovirus or with adenoviruses expressing Syk for the indicated times. Result is from two independent experiments performed in triplicate, shown with SD. (B) Immunoblots of cellular lysates corresponding to cells transduced with control or with Syk-expressing adenoviruses. (C) Cell-cycle distribution of Syk-expressing and control populations as determined by BrdU incorporation and DNA-content flow cytometry analysis. Four days post-infection, cells were pulsed with BrdU and stained with anti-BrdU-APC to detect BrdU incorporation (vertical axis) and 7-AAD to detect total DNA (horizontal axis). The upper box indicates cells in S phase, the lower-left box identifies G0/G1 cells, and the lower-right box displays cells in G2/M. The percentage of cells in the different phases of the cell cycle is indicated. (D) Expression of Syk decreases S phase population. Percentage of BrdU positive cells in A375 transduced with control or Syk expressing adenoviruses. Data are mean \pm SD of three separate experiments.

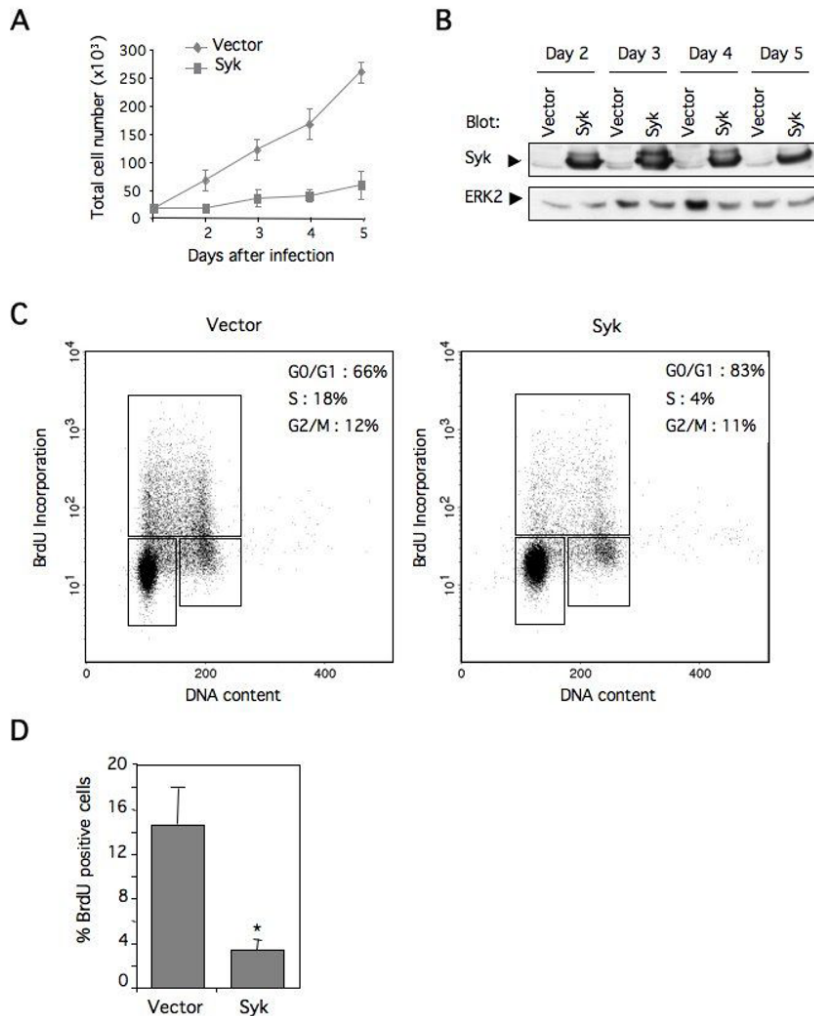


Figure 4

Syk triggers senescence-like phenotypes

(A) Phase-contrast images of A375 cells infected for 6 days with empty vector or with adenovirus encoding Syk. Syk-transduced cells show a large and flat morphology with increased cytoplasmic granularity. The figure shown is a typical representation of the experiment that was performed at least five times (B) Induction of SA- β -gal activity in A375 cells by Syk. Cells were fixed and stained for SA- β -gal (pH 6.0) after 6 days of infection. Upper panels are low-magnification images and lower panels are high-magnification images. (C) Percentage of SA- β -gal-positive cells 7 days post-infection. At least 200 cells were scored from multiple randomly selected fields. Values represent the mean \pm SD of three independent measurements. (D) Syk induces senescence-associated heterochromatin foci (SAHF). A375 cells were stained by immunofluorescence with anti-Syk (red) and anti-HP1 β (green) 6 days post-infection and viewed under a fluorescence microscope. The percentage of SAHF positive cells is indicated in insert. Numbers given are representative of three independent experiments.

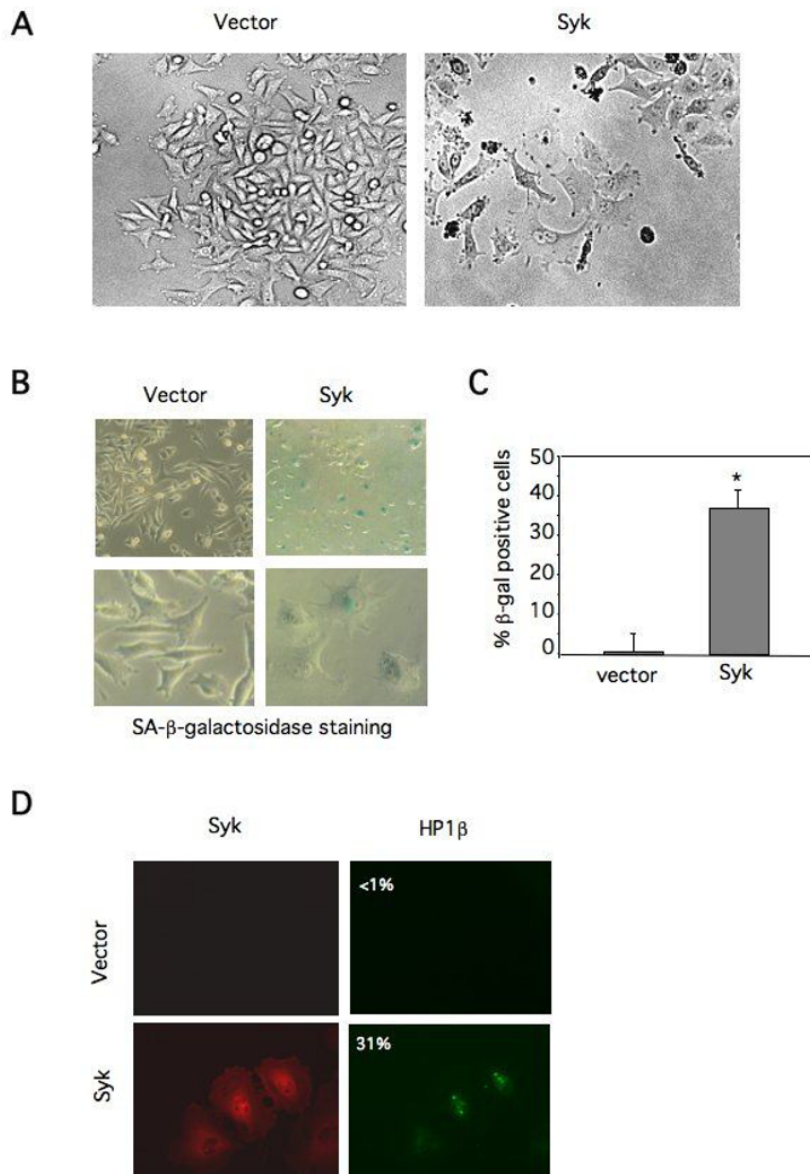


Figure 5

Syk regulates expression and activity of senescence-associated markers

(A) Lysates from A375 infected for 4 days with empty vector or with adenovirus encoding Syk were subjected to immunoblotting with the antibodies indicated on the left. (B) Immunofluorescent staining of vector- and Syk-expressing A375 cells. After 4 days of infection, cells were stained with anti-p53 antibody (green), anti-Syk antibody (red) or DAPI (blue). A treatment for 4 hr with bleomycin was used as positive control. Boxed regions correspond to larger magnifications of representative cells. The percentage of Syk-positive cells showing nuclear labeling of p53 is indicated in insert and is representative of three independent experiments.

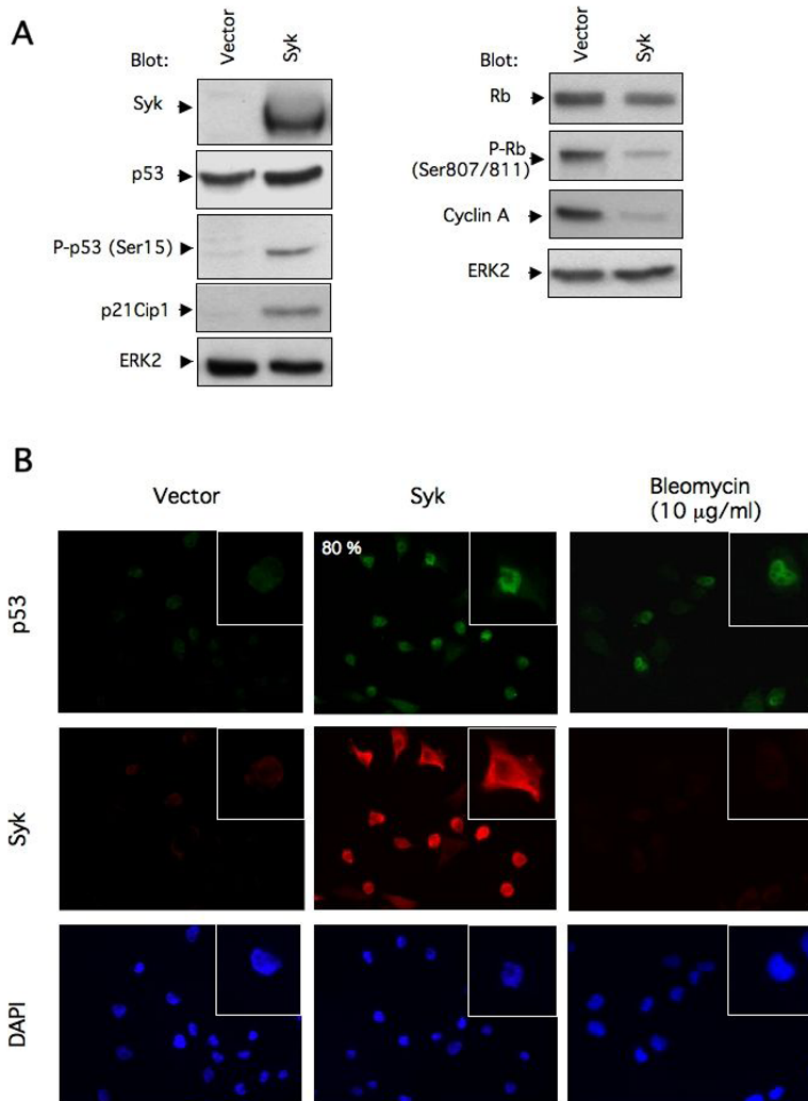
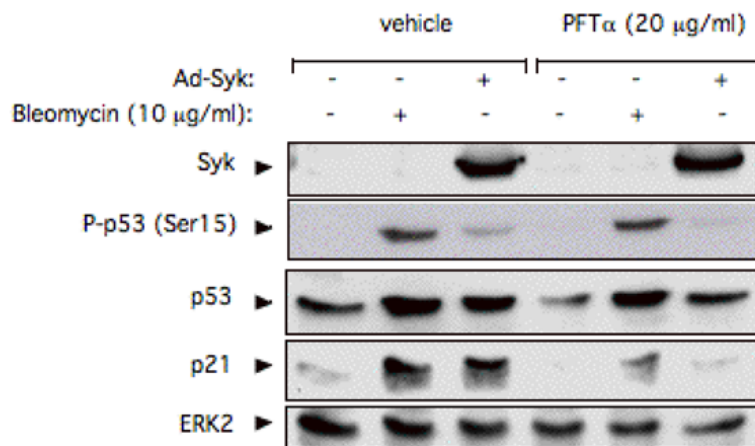


Figure 6

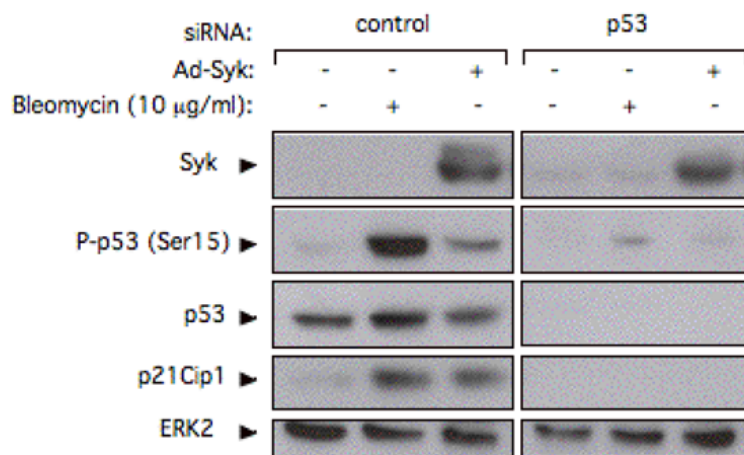
Induction of the cdk inhibitor p21 by Syk expression is p53 dependent

(A) A375 cells infected with empty vector or with adenovirus encoding Syk for 2 days were incubated with pifithrin α (PFT α) (20 μ g/ml) or DMSO as a vehicle control for additional 2 days. Cell lysates were tested for Syk, p53, Phospho (Ser15) p53, p21 and ERK2 by immunoblotting. (B) A375 cells were transfected with p53-siRNA or luciferase-siRNA (control). 6 hr later, cells were infected with empty vector or with adenovirus encoding Syk for 4 days. A treatment with bleomycin was used as positive control. Total cell lysates were subjected to immunoblotting analysis as in A). (C) MeWo (p53 defective) human melanoma cells were infected with empty vector or with adenovirus encoding Syk for 4 days. Cell lysates were tested for Syk, p53, p21 and ERK2 by immunoblotting.

A



B



C

