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The peptidyl prolyl cis/trans isomerase Pin1 downregulates the Inhibitor of Apoptosis Protein Survivin

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Abstract

The peptidyl prolyl cis–trans isomerase Pin1 and the Inhibitor of Apoptosis Protein (IAP) Survivin are two major proteins involved in cancer. They both modulate apoptosis, mitosis, centrosome duplication and neuronal development but until now no functional relationship has been reported between these two proteins. We tested Pin1-induced regulation of Survivin in neuroblastoma cells. Pin1 overexpression in SY5Y neuroblastoma cells decreased Survivin levels. Immunocytochemical studies indicated that they partially co-localized in interphase and mitotic cells. Co-immunoprecipitation further demonstrates the existence of a Pin1/Survivin complex. Pin1-induced effect on Survivin was confirmed in COS cells. RT-PCR and mutagenesis experiments suggested that this Pin1-induced decrease of Survivin occurred at the protein level. Survivin downregulation depended on the binding ability of Pin1 but was not related to the single Thr–Pro site, suggesting an indirect relationship into a protein complex. Finally, this functional regulation of Survivin by Pin1 is reciprocal since Pin1 silencing led to an increase in Survivin levels. The characterization of this functional relationship between Pin1 and Survivin might help to better understand mitosis control and cancer mechanisms.

Keywords: Cell cycle; Mitosis; Neuroblastoma; WW domain; PPIase; Cancer

1. Introduction

The peptidyl–prolyl cis/trans isomerase Pin1 recognises specific motifs consisting of a phosphorylated Ser or Thr residue preceding a Pro (p(Ser/Thr)–Pro motifs) [1,2]. It catalyses the conformational change of the peptide bond between cis and trans conformations. Pin1 has two domains, a N-terminal type IV WW domain and a C-terminal catalytic domain. The WW domain is a protein interacting domain that targets Pin1 to its substrates, whereas the catalytic domain isomerises specific pSer/Thr–Pro motifs [3].

Pin1 is of particular interest in pathogenesis of human diseases, most notably cancer and Alzheimer's disease. A hallmark of the latter is the aggregation of hyperphosphorylated forms of the microtubule associated Tau protein. Pin1 was first described as a new regulator of phosphorylation and conformation of Tau proteins [4]. *In vitro* Pin1 binds to and isomerises pThr–Pro motifs, facilitating dephosphorylation of these motifs in trans-conformation by PP2A [5–8]. In situ, in SY5Y neuroblastoma cells and primary cortical mouse culture, Pin1 facilitates a differential dephosphorylation of Tau at pThr231 [9,10]. Furthermore, some hypotheses state that Pin1 could regulate some cell cycle markers which are altered in Alzheimer's disease [11,12]. In fact, Pin1 induces Cyclin D1 expression at the transcriptional and protein levels [13–16]. This could favour cell cycle entry for proliferating oncogenic cells or re-entry for differentiated neurons. Pin1 also regulates a large number of mitosis-specific phosphoproteins in proliferating cells, many phosphorylated by Cdc2 [17,18]. Pin1 deletion leads to mitotic block and apoptosis [19]. Therefore, Pin1 could help with co-ordinating mitotic events by regulating the function of mitotic phosphoproteins.

Survivin is another major protein involved in mitosis control. Survivin expression increases during the G2-M phases and it is used as a G2-M cell cycle marker [20,21]. It is part of a chromosomal passenger complex which controls chromosome alignment, sister chromatid segregation and cell division [22].

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Moreover, Survivin, like Pin1, is a major protein involved in cancer, especially because, as a member of the Inhibitor of Apoptosis Protein (IAP) family, it can inhibit apoptosis [23]. Pin1 has also been implicated in apoptosis by interacting with proteins like p53 or BIM_{EL} [24–26]. Finally both proteins are involved in brain development. Survivin deletion in early brain development is lethal with massive loss of neurons [27]. Pin1 increases during neuronal differentiation and neurons are the only known differentiated cells where Pin1 is expressed [9].

These similarities between Pin1 and Survivin have led us to investigate their relationships. First, the effect of Pin1 over-expression on Survivin was investigated in a new Pin1-inducible SY5Y cell model. Immunocytochemistry and immunoprecipitation experiments were carried out to study co-localization of both Survivin and Pin1 in neuroblastoma cells. Finally, both Pin1 binding ability and Pin1-induced modulation of Survivin were dissected in COS cells by transfection of different cDNA constructs and Pin1-directed shRNA, proteasome inhibitor treatment and RT-PCR.

2. Materials and methods

2.1. Cell culture and transfection

Pin1 inducible human neuroblastoma cells were generated as described in [10] from native neuroblastoma SH-SY5Y cells. For induction of Pin1 expression, cells were maintained in medium with tetracycline at 1 μg/mL.

Pin1 and Survivin human cDNA [10,28] were subcloned into pcDNA4To (Invitrogen) and transfected using ExGen500 (Euromedex, France). COS-7 cells were grown as previously described. Transient transfection was performed in six-well plates. Mutant Pin1 Ser16Glu and Survivin Thr34Ala were generated by PCR using the quikChange™ Site-Directed Mutagenesis kit (Stratagene) with the forward primer 5′-GG GAG AAG CGC ATG GAG CGC AGC TCA GGC CG-3′, the reverse primer 5′-CG GCC TGA GCT GCG CTC CAT GCG CTT CTC CC-3′ and the forward primer 5′-GCC TGC GCC CCG GAG CGG ATG GCC-3′, the reverse primer 5′-GGC CAT CCG CTC CGG GGC GCA GGC-3′ respectively. A GFP-expressing plasmid was transfected concomitantly to the cells to estimate transfection efficiency. When necessary, an empty pcDNA4 vector was used to cotransfect the same amount of DNA in each condition.

Proteasome inhibition was carried out with epoxomicin (Calbiochem). Conditions were determined by testing 0.5 μM, 1 μM during 12 h and 24 h. Finally we performed the experiments at 1 μM for 12 h.

A siRNA sequence from a set of human Pin1 siRNA duplexes (siGenome duplexes MQ-003291-02-05, DHARMACON) was used as a basis for designing a human Pin1-directed shRNA. Complementary oligonucleotides were annealed and inserted into a linearized shRNA expression vector (RNAi-Ready pSIREN-Shuttle from BD Biosciences). A negative control shRNA annealed nucleotide (PT3739-1, BD Biosciences) was used to construct the control vector.

2.2. Western blotting

Neuroblastoma cells were harvested with Versene (EDTA, Gibco) and centrifuged. Cell pellets were lysed in ice-cold modified RIPA buffer (50 mM Tris, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.5% DOC, 0.1% SDS) containing protease inhibitors (Complete mini; Roche), 1 mM Na_3VO_4 and 125 nM okadaic acid (Sigma). The samples were sonicated and stirred 1 h at 4 °C. Cell lysate was recovered in supernatant after centrifugation at 12,000×*g* at 4 °C for 20 min. Protein concentration was determined by the BCA protein assay kit (Pierce). Samples were mixed with an equal volume of 2× Laemmli buffer and 40 mM dithiothreitol, heated for 5 min at 100 °C and 10 μg were loaded onto SDS-PAGE gel. Transiently transfected COS-7 cells were scraped directly in 100 μL 1× Laemmli buffer and 20 mM dithiothreitol, sonicated and heated. 10 μL was loaded onto SDS-PAGE gel. Electrophoresis and transfer on nitrocellulose membrane (Amersham) were done with the NuPAGE® electrophoresis system, using the Xcell *SureLock*™ Mini-Cell and the Xcell II™ Blot Module (Invitrogen). 4–12% Bis–Tris gels were used. Membranes were blocked in TBST (Tris Buffer Saline Tween, 10 mM Tris–HCl pH 8, 150 mM NaCl, 0.05% Tween 20) with 5% skimmed milk and incubated with primary antibody. Horseradish peroxidase-conjugated antibody (Sigma) was used as secondary antibody, and horseradish peroxidase activity was detected with the ECL detection kit (Amersham Biosciences). Western blots were quantified by densitometry with the IMAGE-MASTER 1D ELITE software (Amersham Biosciences). Results were expressed as means±S.E.M of at least three independent experiments. Statistics were performed using *t* tests with two-tail *P* values.

2.3. Antibodies

Survivin monoclonal antibodies (6E4 Cell Signaling Tech., Ozyme, France, D-8, Santa Cruz Biotech, Tebu-Bio, France), Survivin polyclonal antibody (FL-142, Santa Cruz Biotech), Cyclin D rabbit polyclonal antibody that recognises Cyclin D1 (#06-137, Upstate, Euromedex, France), Neuronal Specific γ-Enolase (NSE) polyclonal antibody (Santa Cruz Biotech), Pin1 polyclonal antibody (H123, Santa Cruz Biotech), GAPDH polyclonal antibody (FL-335, Santa Cruz Biotech), GFP monoclonal antibody (B-2, Santa Cruz Biotech).

2.4. Immunocytochemistry

Cells were fixed in a 4% paraformaldehyde PBS solution 30 min at room temperature. After washing with PBS, cells were permeabilized in 0.25% Triton x100 PBS solution 10 min at room temperature. After washing, non-specific binding sites were blocked in PBS containing 2% bovine serum albumin 1 h at room temperature. Primary antibody was then added in bovine serum albumin solution for 2 h at room temperature. After washing, cells were incubated with the secondary antibody (Alexa Fluor 488® Molecular Probes) in PBS for 45 min. After washing, nuclei were stained with DAPI (1 μg/mL in PBS) for 5 min. Samples were then mounted using Vectashield mounting medium and viewed with a fluorescent optical microscope (LEICA DMRB), a fluorescent confocal microscope (LEICA TCS NT) and by ApoTome (Carl Zeiss). Z-sections are shown. Specificity of the secondary antibody alone was checked.

2.5. Immunoprecipitation

Cell lysates (Pin1 expressing cells treated 48 h with tetracycline) were prepared as described under "Western blotting" but with NP-40 lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40). 1 mg of cell lysates (200 μL at 5 mg/ml) was incubated with the immunoprecipitating antibody (2 μL Survivin 6E4 and 0.6 μg Survivin D-8) overnight at 4 °C and then incubated 2 h at 4 °C with 20 μl of anti-mouse IgG beads (TrueBlot Ig Ip Beads, eBioscience). Immunoprecipitated complexes were washed three times with lysis buffer (centrifugation at 1000×*g* at 4 °C for 5 min), recovered in 25 μL of 2× Laemmli buffer with 100 mM of fresh dithiothreitol, boiled for 5 min, and then loaded onto SDS-PAGE gel and analyzed by immunoblotting.

2.6. Flow cytofluorometry analysis

Cells were harvested with Versene in PBS. A fraction was analyzed by western blotting to check for Pin1 overexpression. Cells were fixed by adding −20 °C absolute ethanol (ratio 5/1 with PBS). After washing with PBS, RNA was degraded by treatment with 1 unit/mL RNAse A (Sigma) for 15 min at room temperature. Cells were permeabilized by triton x100 0.1%, stained with 50 μg/mL propidium iodide for 45 min and analyzed by a fluorescence-activated cell sorter (Epics Altra Beckman Coulter). Results were processed by Expo 32 software (Beckman Coulter) and Multicycle AV (Phoenix Flow system).

2.7. RT-PCR

Purification of mRNA was performed using the NucleoSpin® RNA II kit (Macherey-Nagel) from 6 well dish of transfected COS cells. The reverse

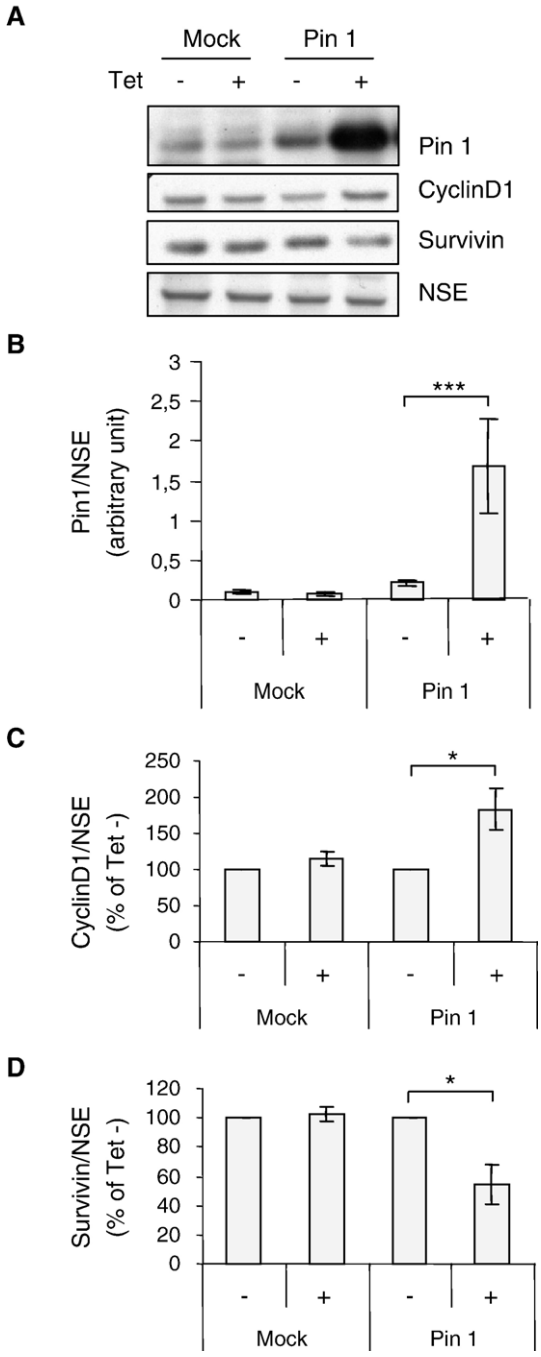

Fig. 1. Effect of Pin1 overexpression on Survivin levels in neuroblastoma cells. (A) Western blots using anti-Pin1, anti-Cyclin D1, anti-Survivin 6E4 and anti-NSE antibodies of cell lysates from mock or Pin1 inducible SY5Y cells in absence (Tet −) or presence (Tet +) of tetracycline for 48 h. (B) Tetracycline treatment induced a Pin1 overexpression in inducible cells. (C) Functional effect of Pin1 overexpression was verified with Cyclin D1 immunostaining. (D) Survivin levels were decreased when Pin1 was overexpressed. NSE immunolabelling is used as an internal loading control for the quantification (*$p<0.05$; ***$p<0.001$).

transcriptase step was done using the B*ca*BEST™ RNA PCR kit (Takara) and semiquantitative PCR with the DyNAzyme™ EXT polymerase (Finnzyme) under experimental conditions allowing the linearity of amplifications, using the forward primer 5′-ATGGGTGCCCCGACGTTGCCC-3′ and the reverse primer 5′-TCAATCCATGGCAGCCAGCTGCTCG-3′ for Survivin and the forward primer 5′-AAACGGCTACCACATCCAAG-3′ and the reverse primer 5′-CGCTCCCAAGATCCAACTAC-3′ for 18S. PCR products were separated on 2.5% agarose gels and bands were quantified by densitometry as described above.

3. Results

3.1. Modulation of Survivin levels in Pin1-expressing SY5Y cells

The expression of Pin1 was analyzed in mock SY5Y and Pin1-inducible SY5Y neuroblastoma cells. As seen in Fig. 1A and B, Pin1 was only slightly detected in mock SY5Y cells. Pin1 inducible cells displayed a low basal expression of transgene protein. Conversely, tetracycline treatment induced a strong Pin1 overexpression in these cells.

Pin1 is well known to stabilize Cyclin D1 in mitotic cells [11,13]. To validate the Pin1 inducible cell model, Cyclin D1 levels were investigated. Pin1 overexpression increased Cyclin D1 immunoreactivity by 75% validating the present cell model (Fig. 1A, C). In the same conditions, Survivin levels were statistically decreased by 50% suggesting an inverse correlation (Fig. 1A, D). To ascertain the specificity of Survivin decrease, two different monoclonal antibodies and one polyclonal antibody were tested. All antibodies showed a Pin1 overexpression-induced Survivin decrease (Fig. 2). Since Survivin is a G2/M marker, we investigated if Pin1 overexpression alters

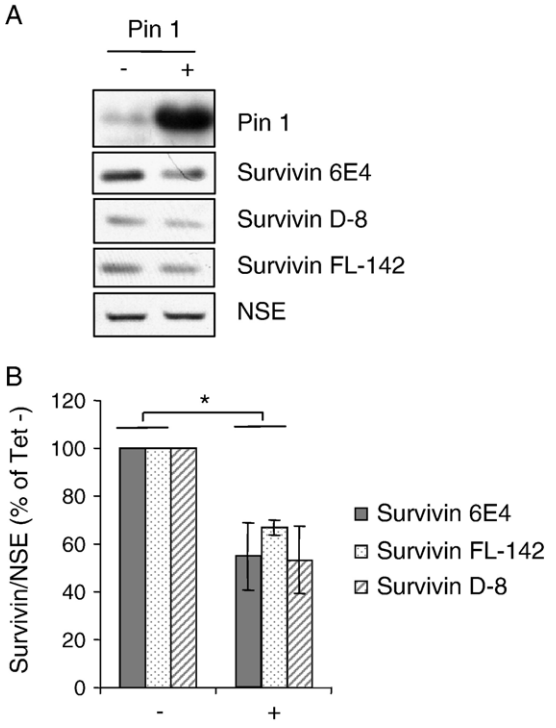

Fig. 2. Survivin labelling specificity. (A) Western blots using anti-Pin1, anti-Survivin 6E4, anti-Survivin D-8, anti-Survivin FL-142 and anti-NSE antibodies of cell lysates from mock or Pin1 inducible SY5Y cells in absence (Tet −) or presence (Tet +) of tetracycline for 48 h. (B) NSE immunolabelling is used as an internal loading control for the quantification (*$p<0.05$).

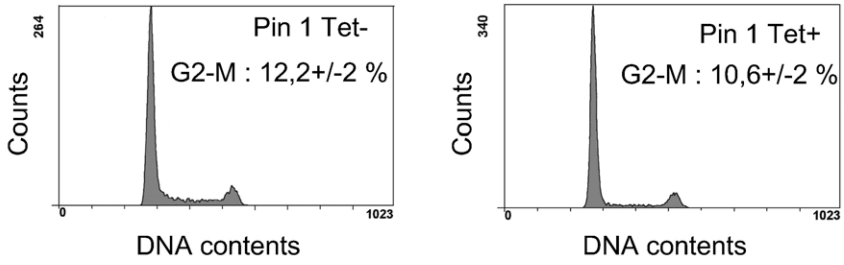

Fig. 3. Pin1 effect on G2-M phases in neuroblastoma cells. Cell cycle repartition of inducible Pin1 cells in absence or presence of tetracycline treatment for 48 h was determined by flow cytometry. G2-M phase cell number was not statistically different.

mitosis progression, as reported in HeLa cells [19]. In the present work, no significant change could be seen by flow cytometry in the G2-M repartition of Pin1 overexpressing neuroblastoma cells (Fig. 3).

3.2. Pin1 and Survivin interactions

To investigate the distribution of Pin1 and Survivin in SY5Y neuroblastoma cells, both proteins were analyzed by immunocytochemistry (Figs. 4 and 5). A weak Survivin labelling was detected in nucleus and cytoplasm of interphase cells (Fig. 4B). The anti-Pin1 antibody labelled the nucleus and more slightly the cytoplasm (Fig. 4A). Ten to 20% co-localization could be observed in the nucleus and cytoplasm of interphasic cells by ApoTome and confocal microscopy. Moreover anti-Survivin antibody sharply stained mitotic cells in comparison with interphasic cells (Fig. 5A2). Chromosomes of prophase and metaphase (Fig. 5B3), central spindle of anaphase (Fig. 5C3) and midbodies of telophase (Fig. 5D3) were typically labelled, supporting a specific labelling of Survivin [29]. Pin1 antibody stained the whole cell except the chromosomes (Fig. 5B2, C2, D2). Colocalization was observed on the central spindle in anaphase (Fig. 5C4) and on the midbodies of telophase (Fig. 5D4).

Since co-localization was observed between Pin1 and Survivin, we investigated a potential interaction by carrying out immunoprecipitation experiments. Survivin was immunoprecipitated with two different anti-Survivin antibodies in Pin1-inducible SY5Y cells (Fig. 6). Pin1 was detected in both immunoprecipitates. Survivin or Pin1 were not detected in control immunoprecipitation of lysate without anti-Survivin antibody. This result suggested that both Survivin and Pin1 could be part of a common protein complex.

3.3. Pin1 Ser16–Glu mutation impairs Pin1-induced Survivin decrease in COS cells

To further investigate the interaction of Pin1 and Survivin, different cDNA constructs were transfected in COS cells. Pin1 and Survivin cDNAs co-transfection significantly decreased Survivin levels (Fig. 7A lane 2), compared to transfection of Survivin cDNA alone (Fig. 7A lane 1). These results confirmed Pin1-induced Survivin reduction observed in SY5Y. Pin1 capacity to bind its substrates was further studied by mutating Ser 16

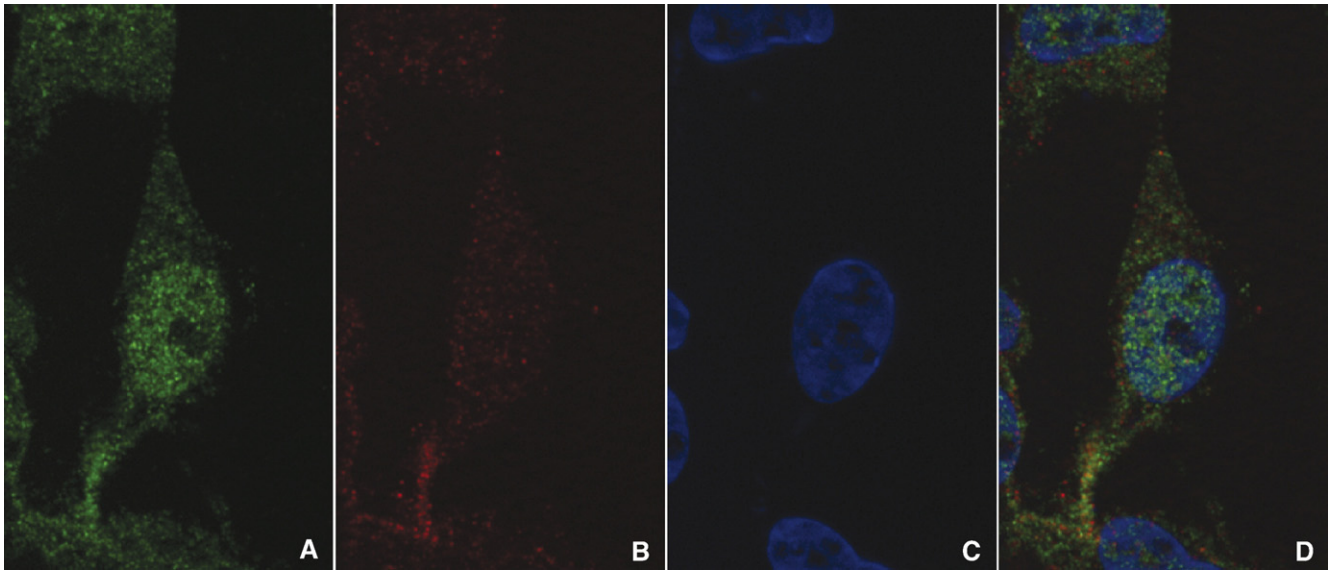

Fig. 4. Co-localization of Pin1 and Survivin in SY5Y interphasic cells. Double immunostaining with anti-Survivin 6E4 and anti-Pin1 H123 antibodies of SY5Y cells observed by ApoTome. (A) Pin1 labelling, (B) Survivin labelling, (C) DAPI labelling, (D) merge.

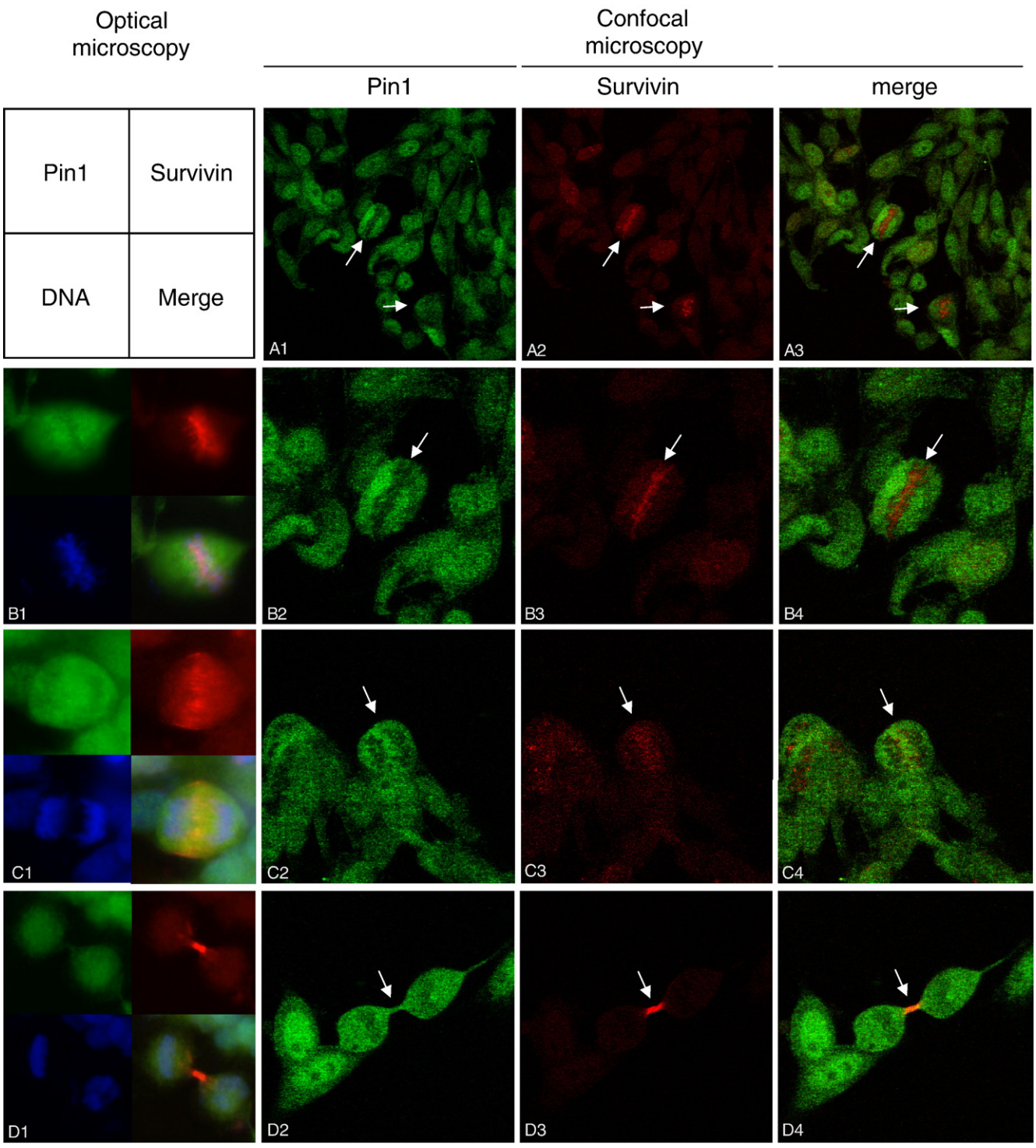

Fig. 5. Co-localization of Pin1 and Survivin in SY5Y mitotic cells. Double immunostaining with anti-Survivin 6E4 and anti-Pin1 H123 antibodies of SY5Y cells. (B1), (C1) and (D1) were observed by optical microscopy whereas the others were observed by confocal microscopy at low magnification (A) or high magnification (B–D). For (B1), (C1) and (D1), top left Pin1 labelling, top right Survivin labelling, bottom left DNA (DAPI labelling), bottom right merge. (A1), (B2), (C2) and (D2) Pin1 labelling, (A2), (B3), (C3) and (D3) Survivin labelling and (A3), (B4), (C4) and (D4) merge. (A) shows an asynchronous fields, where mitotic cells (arrow) are more labelled by Survivin antibody than interphase cells (A2). (B) In metaphase cells, Survivin labelling was high and mainly on chromosomes (arrow and B1). Pin1 did not co-localize on chromosomes. (C) In anaphase cells, Survivin antibodies no more labelled chromosomes but the central spindle (arrow) as well as Pin1 antibodies. Pin1 stained the whole cytoplasm except chromosomes. (D) In telophase/cytokinase cells, Survivin and Pin1 colocalized on midbodies (arrow). Pin1 labelling was also localized in the cytoplasm.

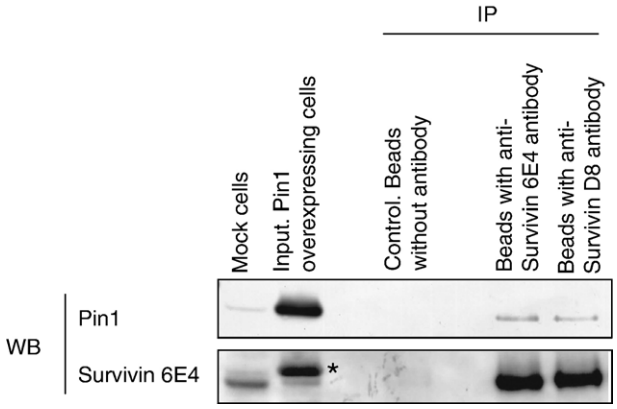

Fig. 6. Co-immunoprecipitation of Pin1 and Survivin in SY5Y cells. Immunoprecipitation of Survivin in a SY5Y cell lysate from Pin1 overexpressing cells (48 h of tetracycline treatment) with anti-Survivin 6E4 and anti-Survivin D-8 antibodies. The blot was probed with Pin1 antibody and then reprobed with anti-Survivin 6E4 antibody. Pin1 is coimmunoprecipitated with Survivin. * Remaining staining of Pin1 during anti-Survivin immunoblotting.

into Glu and the consequences on Survivin levels were analyzed. In fact, glutamate mimics the phosphorylation state of Ser16 and prevents Pin1 binding to its targets [30]. Co-transfection of cDNAs coding for Pin1 mutant instead of wild type Pin1 significantly prevented Survivin decrease (Fig. 7A lane 3). This result suggested that Pin1 binding capacity is necessary to modulate Survivin levels.

3.4. SurvivinThr34–Ala mutation does not prevent Pin1-induced Survivin decrease in COS cells

Since Pin1 and Survivin are in the same protein complex, they may interact directly. Interestingly, Survivin possesses only one Ser/Thr–Pro motif located at Thr34–Pro35, which may be the Pin1 binding site. Thr34 is phosphorylated by Cdc2–Cyclin B during G2-M phases [31]. Inhibition of Thr34 phosphorylation reduces Survivin stability [20,32]. Regulation of this pathway facilitates the elimination of cancer cells at mitosis [33,34]. As with Tau or Cdc25 [5], by isomerising pThr34–Pro35 bond, Pin1 could facilitate dephosphorylation of Survivin pThr34 and modulate Survivin stability. To test this hypothesis, we mutated Survivin Thr34 into Ala, a mutant that cannot be phosphorylated. Surprisingly, co-transfection of Pin1 with Thr34–Ala mutant still decreased the level of this mutant (Fig. 7B lane 2). Thus, the functional effect of Pin1 on Survivin levels is likely to be unrelated to a direct physical interaction between the two proteins.

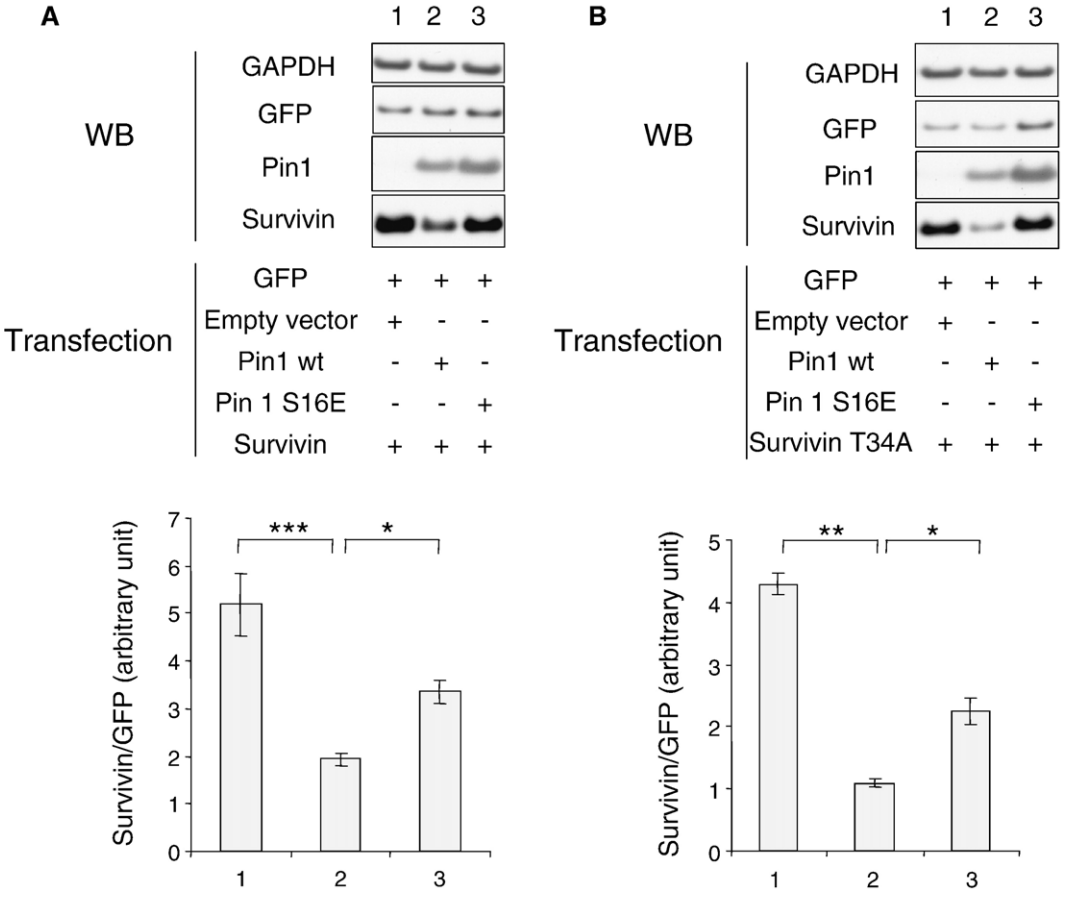

Fig. 7. Co-transfection of Pin1 and Survivin in COS cells. (A) Western blotting of cell lysates. COS cells were transfected with cDNAs coding for Survivin alone (lane 1), Survivin and wild type Pin1 (lane 2) and Survivin and mutated Pin1 S16E (lane 3). GFP was cotransfected each time as transfection control. GAPDH is used as a loading control. Co-transfection of cDNAs coding for both Pin1 and Survivin decreased Survivin protein level. Co-transfection of cDNAs coding for Survivin and mutated Pin1 altered Survivin protein level but to a lesser extent. (*$p<0.05$; ***$p<0.001$). (B) The same experiment was carried out with mutated Survivin Thr34Ala. Results were similar (*$p<0.05$; **$p<0.01$).

3.5. Effect of Pin1 overexpression on Survivin mRNA levels and on proteasome-mediated Survivin degradation in COS cells

Since the interaction is likely to be indirect, we investigated how Pin1 could regulate Survivin levels. Pin1 has already been shown to regulate mRNA stability [35] and transcription by interacting with RNA polymerase II [36]. To check if Pin1-induced Survivin regulation occurred at the transcription level, Survivin mRNA amount was analyzed by semi-quantitative RT-PCR (Fig. 8A). Pin1 and Survivin cDNA co-transfection did not lower Survivin mRNA (Fig. 8A lane 2), suggesting that Pin1-induced Survivin protein decrease is not related to changes in mRNA levels. Thus, Pin1-induced Survivin regulation is likely to occur at the protein level.

Survivin is known to be degraded by the proteasome [37]. To test if Pin1 could regulate Survivin level by acting on its

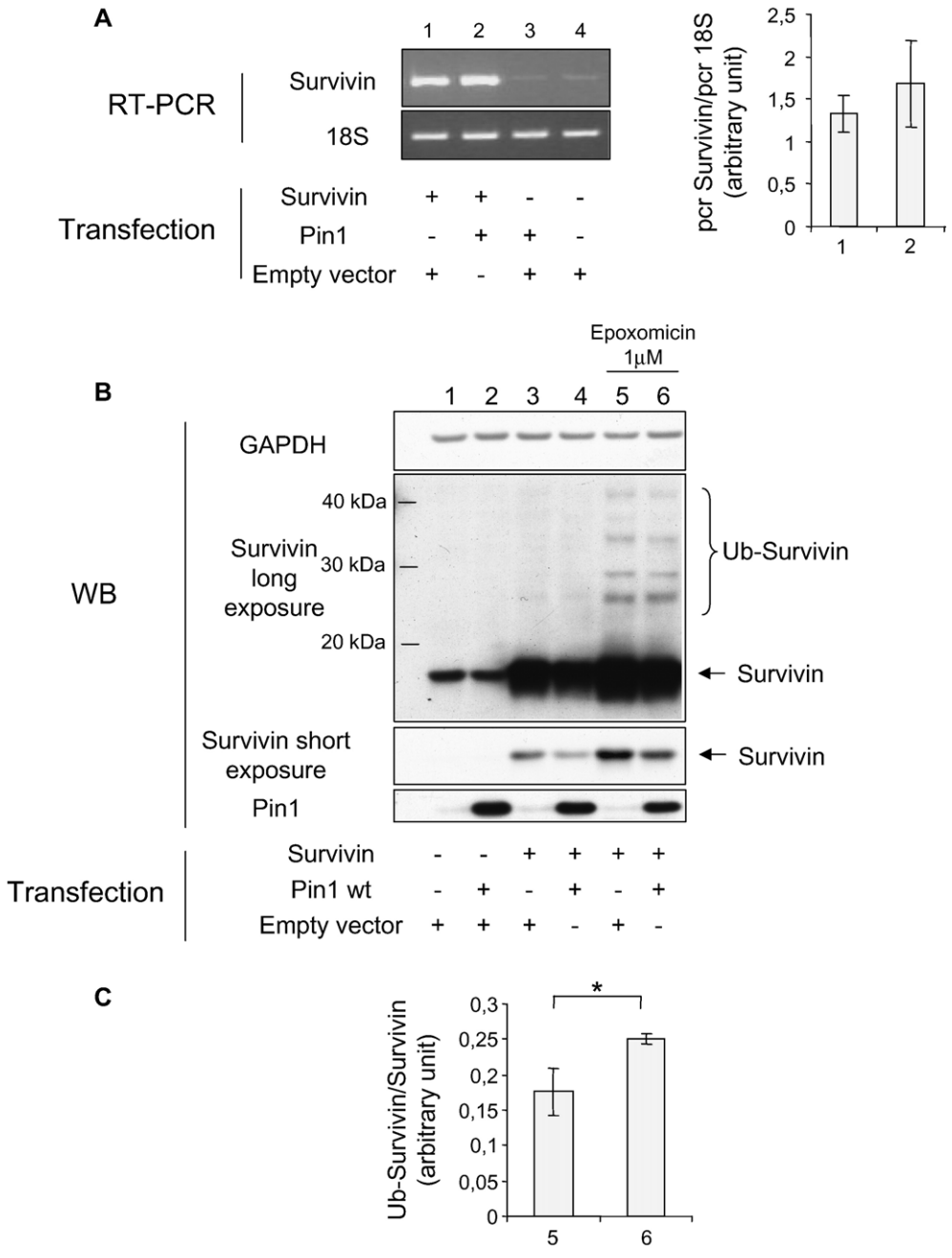

Fig. 8. Effect of Pin1 overexpression on Survivin mRNA and on Survivin degradation by the proteasome in COS cells. (A) COS cells were transfected with cDNAs coding for Survivin alone (lane 1), Survivin and Pin1 (lane 2), Pin1 alone (lane 3) and with an empty vector (lane 4). Co-transfection of cDNAs coding for both Pin1 and Survivin did not alter Survivin RNA levels. (B) Western blotting of cell lysates. COS cells were transfected with an empty vector (lane 1), with cDNAs coding for Pin1 alone (lane 2), Survivin alone (lane 3 and 5), Pin1 and Survivin (lane 4 and 6). At the end of the transfection, epoxomicin (1 μM) was added to the medium (lane 5 and 6). Epoxomicin treatment increased Survivin and ubiquitinated Survivin levels but did not abolish Pin1 overexpression-induced Survivin decrease. Nevertheless, the ratio of ubiquitinated Survivin out of Survivin increased in Pin1 overexpression condition (lane 6) ($*p<0.05$).

degradation by the proteasome, we treated transfected COS cells with the proteasome inhibitor epoxomicin (Fig. 8B) [38]. Proteasome inhibition led to an increase of Survivin and ubiquitinated Survivin levels in the presence or absence of Pin1 overexpression but could not completely abolish Pin1 overexpression-induced Survivin decrease. However, when treated with epoxomicin, the ratio ubiquitinated Survivin on Survivin was increased when Pin1 was overexpressed (Fig. 8B lane 6 versus 5 and Fig. 8C), indicating that Pin1 could partially modulate Survivin regulation by the ubiquitin–proteasome pathway as observed for cyclinE–SCF ubiquitin ligase complexes [39].

If Pin1 facilitates Survivin degradation, one may ask if there is an increase of Survivin in the absence of Pin1.

3.6. Pin1-directed shRNA inhibits Pin1-induced Survivin decrease in COS cells

After Pin1 overexpression, the reciprocal effect of Pin1 silencing on Survivin levels was investigated (Fig. 9). Survivin expression plasmid and Pin1-directed shRNA vector were transfected in COS cells. This co-transfection led to a significant increase of Survivin (Fig. 9 lane 2). Furthermore, co-transfection of the Pin1-directed shRNA vector with both Pin1 and Survivin cDNAs reduced Pin1 overexpression and partially restored Survivin expression (Fig. 9 lane 4). These results confirm a negative correlation between Pin1 and Survivin levels. All together, it suggests that Pin1 can modulate Survivin levels.

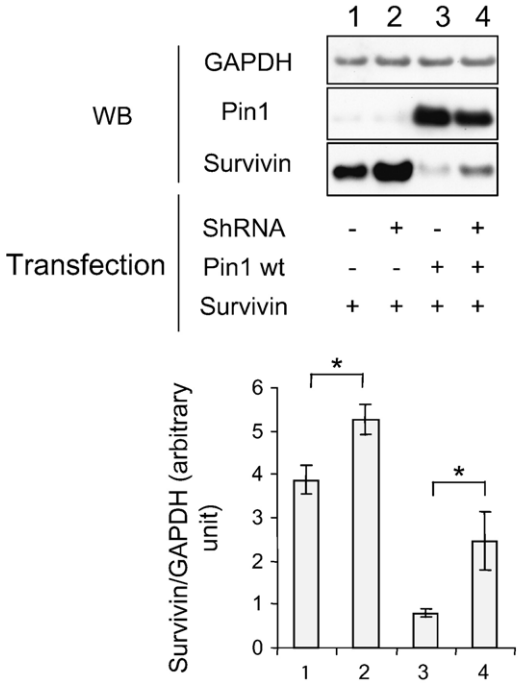

Fig. 9. Effect of Pin1 silencing on Survivin level in COS cells. Western blotting of cell lysates. COS cells were transfected with cDNAs coding for Survivin alone (lane 1), Survivin and Pin1-directed shRNA (lane 2), Survivin and Pin1 (lane 3), Survivin Pin1 and Pin1-directed shRNA (lane 4). Co-transfection of cDNA coding for Pin1-directed shRNA decreased overexpressed Pin1 level and increased Survivin protein level. GAPDH is used as a loading control (*$p<0.05$).

4. Discussion

The relationship between Pin1 and Survivin has been investigated in neuronal cells. In SY5Y neuroblastoma cells, overexpression of Pin1 decreased Survivin levels. This effect could be reproduced by transient transfection in COS cells. Conversely, Pin1-directed shRNA increased Survivin levels. Therefore Pin1 expression is able to modulate Survivin levels. These results show for the first time a functional relationship between Pin1 and Survivin, two major proteins involved in human cancers.

Pin1 or Survivin are overexpressed in many tumors [40,41] and are usually negative prognostic factors [42,43]. They are both promising therapeutic targets [44,45]. Pin1 amplifies many pro-proliferative signallings in cancer cells and its inhibition would provide a way of tackling simultaneously multiple oncogenic signal pathways at several levels [46]. Survivin may greatly be involved in the chemo- and radio-resistance of tumour cells and its inhibition could drive cancerous cells to death by increasing their sensitivity to chemical and physical agents [44]. Our results point out that acting on Pin1 might modulate Survivin levels. It should be taken into consideration for therapeutic strategies.

Pin1 has been previously reported to regulate the cellular decrease of various proteins like cyclin E, c-Myc or Pim1 [47–49]. Nevertheless, the negative regulation of Pin1 on Survivin is particularly intriguing. It does not fit with reported overexpression and pro-oncogenic role of both proteins in cancers. However until now there is no evidence showing that Pin1 and Survivin are concomitantly overexpressed in the same tumoral cells. If Pin1-induced decrease of Survivin occurs in neuroblastoma, it should lead to tumour regression. To this point, noticeably, occurrence of neuroblastoma spontaneous regressions has been reported [50]. Recently Pin1 has been shown to increase during neuroblastoma differentiation, suggesting a role of Pin1 overexpression in neuronal differentiation [9]. In neuroblastoma cells, Pin1 increase might have a dual role and promote either oncogenesis or differentiation. Moreover, Pin1 might facilitate physiological cell cycle-related down-regulation of Survivin levels. This mechanism could be altered in cancer favouring Survivin overexpression. Further studies will be necessary to explore these aspects.

Pin1 has been recently shown to regulate GM-CSF mRNA stability [35,51] and transcription by interacting with RNA polymerase II [36]. RT-PCR experiments showed that Survivin downregulation was not due to decreased Survivin mRNA and did not happen at the transcription level. Nevertheless, an effect on endogenous Survivin pre-mRNA cannot be rejected. Our results suggested that Pin1 regulation occurred at least at the protein level. Pin1 mutation of Ser 16 to Glu had an inhibitory effect on Survivin modulation. This mutation inactivated the binding capacity of Pin1 on target proteins. Thus p(Ser/Thr)–Pro recognition is needed for Pin1 overexpression-induced effect. Nevertheless, mutation of the only p(Ser/Thr)–Pro site of Survivin (Thr34–Ala) did not abolish Pin1 overexpression outcome. Pin1–Survivin interaction rather seemed indirect. It is conceivable that Pin1 could indirectly modulate Survivin levels

through binding and isomerisation of a common partner to both proteins.

Co-immunoprecipitation in neuroblastoma cells demonstrated that Pin1 and Survivin could be part of a common protein complex. Partial co-immunolocalization in neuroblastoma cells further illustrated Pin1 and Survivin close relationship. It is worth noting that only a small fraction of Pin1 has been found in complex with Survivin. This result is in accord with the fact that Pin1 only transiently interacts with its substrates. This could also be explained because Pin1 downregulated Survivin.

There are numerous protein complexes where Pin1 and Survivin can be found together with common protein partners. During mitosis, Survivin associates with Aurora B and INCENP on the centromere/kinetochore to form a chromosomal passenger complex [22]. Aurora B and INCENP have been described as Pin1 partners [52]. Aurora B has two Thr–Pro sites [53] including the phylogenetically conserved Thr35 (found in many ortho-/homo-logs with the exception of rodents) which may allow Pin1 binding. In addition, Pin1 has already been related to Plk1 which is involved in the regulation of the chromosomal passenger complex [54,55]. Pin1 and Survivin could also interact at microtubules and centrosome level. Survivin binds to microtubules [21]. Pin1 copurifies with γ-tubulin and localizes to centrosome [56]. Overexpression of Pin1 in mouse mammary glands potently induces centrosome amplification, eventually leading to mammary hyperplasia and malignant mammary tumors with overamplified centrosomes [56]. Pin1 downregulation of Survivin could sustain these results as Survivin colocalizes with caspase3 and $p21^{WAF1/CIP1}$ within centrosome and as interference with Survivin expression or function causes cell-division defect characterized by centrosome deregulation, multipolar mitotic spindles and multinucleated, polyploid cells [57].

In conclusion, the functional interactions between Pin1 and Survivin may be of particular interest in oncogenesis and cell division. Further studies will be needed to better understand the mechanisms by which Pin1 and Survivin interact and their consequences.

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