Phosphorylated Tyr142 β-catenin localizes to centrosomes and is regulated by Syk

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Funding information
Instituto de Salud Carlos III/ Feder “Una manera de hacer Europa” (Spain), Grant numbers: PI08/0790, PI13/01980

Abstract
β-catenin is a central component of adherent junctions and a key effector of canonical Wnt signaling, in which dephosphorylated Ser/Thr β-catenin regulates gene transcription. β-catenin phosphorylation at Tyr142 (PTyr142 β-catenin), which is induced by receptor and Src family Tyr kinases, represents a previously described β-catenin switch from adhesive to migratory roles. In addition to classical β-catenin roles, phosphorylated Ser/Thr β-catenin and total β-catenin were involved in centrosomal functions, including mitotic spindle formation and centrosome separation. Here we find that PTyr142 β-catenin is present in centrosomes in non-transformed and glioblastoma cells and that, in contrast to the Ser/Thr phosphorylated β-catenin, PTyr142 β-catenin centrosomal levels drop in mitosis. Furthermore, we show that the inhibitor of Spleen Tyrosine Kinase (Syk) piceatannol decreases centrosomal PTyr142 β-catenin levels, indicating that Syk regulates centrosome PTyr142 β-catenin. Our findings suggest that PTyr142 β-catenin and Syk may regulate centrosomal cohesion. This study highlights the contribution of different phosphorylated β-catenin forms to the cell and centrosome cycles.

KEYWORDS
β-catenin, centrosome, glioblastoma, piceatannol, syk

1 | INTRODUCTION

Centrosomes are microtubule (MT) organizing centers that control cell polarity, adhesion, motility, and cytokinesis. The two centrosomes present at the onset of mitosis determine the proper formation of bipolar MT spindles that control chromosome segregation. Thus, alterations in centrosome functions and number contribute to chromosome instability and are frequent in cancer.1,2

A centrosome consists of two centrioles surrounded by pericentriolar matrix (PCM), where γ-tubulin-ring complexes act as MT-nucleating templates. Centrosome structure and number are regulated through the cell cycle. During mitosis, the centrosome at each pole of the mitotic spindle contains a pair of tightly connected centrioles. However, this association is lost at the end of mitosis, leading to the separation of the daughter cells. Centriole duplication occurs in S phase, with pro-centrioles that elongate and function as a single MT-organizing center until late G2. At G2-M transition, centrosome maturation involves the exchange of PCM components and further recruitment of γ-tubulin complexes. In response to activated MT-dependent motor proteins, centrosomes finally separate and instruct the formation of spindle poles that ensure that each daughter cell inherits one centrosome.

β-catenin is a component of cell adhesion complexes and a key Wnt signaling effector. In the cell adhesion complex,
β-catenin dissociation from cadherin or from α-catenin, in part induced by its Tyr phosphorylation, decreases cell adhesion while increasing cell migration. Furthermore, in the canonical Wnt signaling pathway, β-catenin acts as transcriptional co-regulator together with transcription factors of T-cell Factor/Lymphoid Enhancer Factor families. Briefly, when signaling is off, β-catenin taking part of the destruction complex is phosphorylated by Glycogen Synthase Kinase (GSK)-3β at Ser33/37/Thr47, which is then degraded by the proteasome. In contrast, in the presence of Wnt, β-catenin (dephosphorylated at Ser33/37/Thr47) accumulates and regulates Wnt target expression. Interestingly, β-catenin phosphorylation at Tyr142 (PTyr142 β-catenin) implies a switch from adhesive to transcriptional functions independent of Wnt.4,5 which is regulated by Met and Hepatocyte Growth Factor signaling5,6 and other Tyr kinases.7 PTyr142 β-catenin therefore dissociates from the adhesion complex, localizes to the nucleus and promotes cell migration and axon growth in neurons.5,6

While the classical roles of β-catenin both in cell adhesion and Wnt signaling are well known, the mechanisms involving centrosome β-catenin functions are less understood. β-catenin found in centrosomes regulates the establishment of a bipolar mitotic spindle and centrosome separation.8–12 Stabilized forms of β-catenin (lacking N-terminal regions that regulate its degradation, mimicking mutations in cancer cells) cause extra non-MT nucleating structures and promote centrosome separation.9,10,12

β-catenin phosphorylated in Ser33/Ser37/Thr41 (PSer/Thr β-catenin; tagged for degradation when Wnt signaling is off) also accumulates in centrosomes,8 where it remains stable during mitosis.13 Expression of phospho-mimetic β-catenin mutants (Ser33/Ser37/Thr41 mutated to Glu) results in multiple centrosomes and aberrant MT arrays,8 indicating that PSer/Thr β-catenin regulates the anchoring of the MT array to the centrosome. PSer/Thr β-catenin levels oscillate during the cell cycle (peaking at G2/M) and parallel those of Axin2, a negative regulator of Wnt signaling.14 Furthermore, centrosomal PSer/Thr β-catenin regulates centrosome cohesion.12 Consistent with this, Wnt signaling, GSK3 inhibitors, stabilized β-catenin or Axin2 knockdown promote centrosome separation.12

Here, we analyze the putative presence of β-catenin phosphorylated at Tyr142 in centrosomes and its regulation. We describe for the first time the centrosome localization of PTyr142 β-catenin in astrocytes and glioblastoma (GBM) cells. Using phosphospecific antibodies, we demonstrate the co-localization/co-fractionation of PTyr142 β-catenin with centrosome markers. Cell cycle analysis indicates that centrosomal PTyr142 β-catenin levels fall in mitosis, paralleling a drop of Syk centrosomal levels reported previously.15 Furthermore, we show that Syk phosphorylates β-catenin at Tyr and that centrosome PTyr142 β-catenin levels decrease upon exposure to the Syk inhibitor picea-tannol (Pic). Thus, we identify a PTyr142 β-catenin centrosomal pool regulated by Syk that, different to PSer/Thr β-catenin,8 vanishes from the centrosome in mitosis.

2 | MATERIALS AND METHODS

2.1 | Cell culture and experimental animal research

U251MG and U87MG GBM cell lines, available from ATCC, were maintained in Minimum Essential Medium (MEM) media containing 10% heat-inactivated foetal bovine serum (FBS), 10% non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 20 U/mL penicillin/streptomycin (P/S).

Investigation with experimental animals was approved by the Experimental Animal Ethics Committee of UdL (CEEA 10-02/08, CEEA 11-02/08) and followed the Helsinki declaration. Animals were anesthetized with a lethal dose of inhaled isoflurane and decapitated within the animal facility.

Embryonic mouse fibroblasts (EMFs) were obtained from 14 day old mouse embryos following dissociation using 0.25% trypsin-EDTA and cultured in DMEM (4.5 g/L glucose), 10% FBS, 2 mM glutamine and 20 U/mL P/S. Rat striatal astrocytes were isolated as described,16 from postnatal day 0-1 rat brains and cultured on poly-L-ornithine (PLO) pre-coated plates (1.5 µg/mL, 1h, 37°C) in Astrocyte Culturing Media (Dulbecco’s Minimum Essential Media 1 g/L glucose, 2 mM glutamine, 20 U/mL P/S and 10% FBS).

2.2 | Cell transfection

For knockdown using siRNA, RNaiMAX Lipofectamine was used. Briefly, 25 µL of Optimem containing siRNA sequences (80 nM; Sigma–Aldrich, St. Louis, MO; VPDSIRNA2D for β-catenin and PDSIRNA2D for GAPDH, used as control), together with 0.2 µg of Green Fluorescence Protein DNA plasmid, were mixed with 25 µL of Optimem plus 1 µL of RNaiMAX Lipofectamine. The mix was incubated for 15 min at room temperature (RT) and added dropwise to cells for 6 h. Then media was changed for complete media (MEM including FBS).

2.3 | Antibodies

Anti-PTyr142 β-catenin antibodies were purchased from Abnova, Taipei, Taiwan; anti-β-catenin from BD Bioscience (San José, CA), or Cell Signaling Technology (Danvers, MA) (N-terminus), anti-γ-tubulin, and anti-α-tubulin from Merck/Sigma–Aldrich, anti-Syk from Santa-Cruz Biotechnology (Dallas, TX) and anti-PTyr (4G10) from Upstate Biotechnology (Lake Placid, NY).
PTyr142 β-catenin is found in centrosomes. A) Immunostaining for PTyr142 β-catenin and the centrosome marker γ-tubulin in rat striatal astrocytes, U251MG, and U87MG GBM cells and mouse embryonic fibroblasts (EMFs). Arrows point at centrosomes doubly immunostained by anti-γ-tubulin and anti-PTyr142 β-catenin antibodies. PTyr142 β-catenin can also be found in the nucleus. Nuclei were stained by Hoechst (H). Bars = 15 μm. B) Immunostaining for PTyr142 β-catenin in control or siRNA β-catenin transfected U251MG cells. Double staining with Hoechst is shown. Bar = 10 μm
2.4 | Cell treatments and immunofluorescence

Cells were plated on Poly-D-Lysine (PDL)-coated (25 µg/mL) or PLO-coated (astrocytes) coverslips. Pic 200 µM was added to cells for 6 h. Pervanadate was prepared as previously described. Mitosis arrest was carried out on cells plated on PDL-coated coverslips in complete media. Cells were deprived of serum for 20 h and then grown in complete media for the 16-20 h before methanol fixation.

Cells were either fixed using 4% Paraformaldehyde (20 min, RT) or ice cold methanol (5 min, −20°C), washed with Phosphate buffered saline (PBS) and blocked/permeabilized with PBS containing 5% FBS, 5% horse serum, 0.2% Glycine, and 0.1% Triton X-100. Triton X-100 was omitted in methanol-fixed cells. Cells were incubated with primary antibodies (overnight, 4°C) and subsequently washed and incubated with Alexa-488 or Alexa-564-coupled secondary antibodies, together with Hoechst. Coverslips were mounted on Mowiol. Images were obtained using an inverted Olympus IX70 microscope (10×, 0.3 numerical aperture (NA); 20×, 0.4 NA; 32×, 0.4 NA) equipped with epifluorescence optics and a camera (Olympus OM-4 Ti) or using a FluoViewTM FV1000 Confocal Microscope (60×). DPM Manager Software was used together with Olympus IX70 microscope, whereas FV10-ASW software was used for confocal images.

Relative immunofluorescence intensities of centrosomal PTyr142 β-catenin from individual cells were measured using the RGB plugin (ImageJ software). Statistical significance was calculated using the Student T test (paired) (***(P ≤ 0.001).

2.5 | Centrosome isolation

Centrosomal isolation was performed as described. Exponentially growing (8 p100 plates) U251MG cells were treated with Nocodazole (0.2 µM) and Cytochalasin D (1 µg/mL), 1 h at 37°C, to depolymerize actin and MTs. Cells were harvested by trypsinisation and lysed in 4 mL lysis buffer: 1 mM Hepes (pH 7.2), 0.5% Igepal, 0.5 mM MgCl2 and 0.1% β-Mercaptoethanol (β-Me) containing Complete protease inhibitors and phosphatase inhibitors (40 mM β-Glycerophosphate, 1 mM Sodium Orthovanadate and 25 mM Sodium Fluoride). Nuclei and chromatin aggregates were removed by centrifugation (5500 rpm, 10 min) and the supernatant filtered (70 µm). A total of 9 mM Hepes and DNAse I (2 U/mL) were added to the lysate and incubated on ice for 30 min. Typically,
4 mL of the lysate were underlaid with 0.5 mL of 60% sucrose solution (60% w/w sucrose in 10 mM Pipes pH-7.2, 0.1% Triton X-100, 0.1% β-Me) and centrifuged (12 500 rpm, 30 min; JA14 rotor, Beckman Coulter (Brea, CA), Avanti J-26 XP centrifuge) to sediment the centrosomes into the cushion. After centrifugation, the top 3 mL were discarded and the remaining 1.5 mL loaded into a discontinuous gradient consisting of: 500 µL of 70% sucrose, 300 µL of 50% sucrose and 300 µL of 40% sucrose in the Pipes buffer using. Tubes (Beckman polyallomer centrifuge tubes) were centrifuged (35 000 rpm, 30 min; TLS55 rotor; Beckman Coulter). Fractions were collected from the top: 500 µL were collected for fraction #8 (top) and the rest of the fractions (# 0-7) of 200 µL/fraction. Fractions were diluted to 1 mL in 10 mM Pipes buffer, pH 7.2, and precipitated with Trichloracetic acid (6.5%, 20 min). Pellets were dissolved in loading buffer plus Tris-HCl, pH 8.

### 2.6 Immunoprecipitation

U87MG cells were lysed in ice-cold IP buffer: 50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Igepal (NP-40), 10% glycerol containing protease and phosphatase inhibitors as above mentioned. β-catenin immunoprecipitation (BD Bioscience antibody) was performed as described, using 500-800 µg of protein (as determined by the Lowry assay). Immunoprecipitated complexes were dissociated from the beads in loading buffer without β-Me.
2.7 | In vitro kinase assay

GST-β-catenin recombinant proteins were produced in BL21 bacteria. GST-β-catenin WT or Y142F proteins (1 µg; 8 pmols) were phosphorylated by recombinant Syk kinase (Precisio™, Sigma–Aldrich) following manufacturer’s instructions (30 min, 30°C) in kinase buffer (5 mM HEPES, pH 7.2, 4 mM MgCl₂, 2.5 mM MnCl₂, 0.4 mM EDTA, 1 mM EGTA, 0.05 mM DTT, 1.5 mM β-glycerophosphate with or without 400 µM Pic or 100 nM Staurosporine and 0.1 mM ATP). Samples were analyzed by western blot using PTyr antibodies.

FIGURE 4 Continued.
2.8 Western blotting

Proteins were resolved by SDS-PAGE and transferred to Immobilon-P using a semi-dry apparatus. Membranes were blocked with Tris-buffered saline plus Tween-20 (TBS-T) (20 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.05% Tween-20) containing 5% non-fat dry milk (1 h, RT), washed with TBS-T and incubated overnight with the primary antibody. Membranes were then incubated with peroxidase-conjugated secondary antibodies before developing, using either Enhanced chemiluminescence (ECL) or Supersignal reagents.

3 RESULTS

Different cell types, including primary rodent fibroblasts, striatal astrocytes and human GBM (U251MG and U87MG) cells were immunostained against PTyr142 β-catenin and γ-tubulin, a centrosome marker. The co-localization of PTyr142 β-catenin and γ-tubulin immunostainings suggested the presence of PTyr142 β-catenin in centrosomes in all tested cell types, including cancerous and non-transformed cells (Figure 1A). This immunostaining was not observed in absence of primary antibody (data not shown) or in β-catenin silenced cells (Figure 1B). To confirm the centrosomal localization of PTyr142 β-catenin, we performed centrosome subfractionation and immunoprecipitation experiments. Centrosome fractions were isolated from U251MG cells, using a discontinuous sucrose gradient centrifugation method. Fractions 2 and 3, corresponding to ~50-60% sucrose and identified by a peak of γ-tubulin, were considered as the centrosome-enriched fractions (Figure 2A). PTyr142 and total β-catenin co-fractionated with γ-tubulin-enriched centrosome fractions at the expected sucrose density (Figure 2A). Syk also co-fractionated with γ-tubulin and β-catenin (Figure 2A), consistent with its centrosome localization. Furthermore, γ-tubulin co-immunoprecipitated with PTyr142 β-catenin in β-catenin immunoprecipitated-complexes (Figure 2B). Together, these results demonstrate that a fraction of PTyr142 β-catenin associates to centrosomes.

We then studied whether centrosome PTyr142 β-catenin is regulated in mitosis. To this end, we used U251MG cells upon cell cycle-arrest induced by serum deprivation for 20 h followed by a period of 16-20 h of release in complete media, in which mitotic figures were captured. Centrosomal PTyr142 β-catenin immunostaining was compared during the different mitotic phases identified by Hoechst (to label the DNA) and α-tubulin (to reveal the MT reorganization) stainings (Figure 3). While PTyr142 β-catenin appeared centrosomal in interphase cells (arrows; Figure 3), PTyr142 β-catenin decreased abruptly in metaphase centrosomes and remained absent from centrosomes until the end of telophase/cytokinesis, when it reappeared at the centrosomes of the daughter cells (Figure 3). These findings indicate that centrosomal PTyr142 β-catenin is lost in mitosis, suggesting that its presence negatively affects mitotic progression.

Next, we sought to investigate which kinase regulates PTyr142 β-catenin at the centrosome. Syk appeared as a possible candidate because it is associated to interphase centrosomes, is removed from centrosomes in mitosis and phosphorylates cell adhesion proteins (E-cadherin and α-catenin). Immuno-staining experiments confirmed that Syk localizes to centrosomes in GBM cells (data not shown), in agreement with subfractionation experiments. Therefore, we performed in vitro kinase assays using recombinant Syk and β-catenin Wild-type (WT) or Y142F. Results indicate that Syk phosphorylates WT β-catenin in Tyr residues, which was inhibited by the prototypical ATP-competitive kinase inhibitor Staurosporine and by the Syk inhibitor Pic (Figure 4A). In addition, PTyr phosphorylation of Y142F β-catenin was reduced compared to that of WT protein, and its phosphorylation level was similar with or without Pic (close to basal phosphorylation levels detected in absence of ATP; Figure 4A).

Next, we tested whether regulating Syk activity could affect centrosomal PTyr142 β-catenin in GBM cells treated with Pic. Centrosomal PTyr142 β-catenin immunostaining decreased in GBM cells treated with Pic versus control cells
(by 40–50% in U251MG and U87MG cells, respectively, according to fluorescence intensity measurements; Figures 4B and 4C). Centrosomal Pγ142 β-catenin fluorescence intensity was not statistically significant when control cells were compared to cells treated with sodium pervanadate, a Tyr phosphatase inhibitor and Syk activator19 (Figure 4C). In addition, U251MG cells treated with Pic showed an increased % of cells displaying two well separated centrosomes compared to control cells (Figure 4D), suggesting that Pγ142 β-catenin may be involved in centrosomal cohesion/separation. Together, these results indicate that Syk phosphorylates centrosomal β-catenin in Tyr142.

4 | DISCUSSION

Although β-catenin's best studied roles include the transcriptional regulation of Wnt targets and cell adhesive functions,21 the association of β-catenin with the centrosome has been known for years,11 when early studies showed its contribution to mitotic spindle formation and centrosome separation.9,11 Mitogenic Wnt/β-catenin signalling regulates cell proliferation, and β-catenin and Wnt pathway components located to the centrosomes assist mitosis.22 Indeed, in addition to β-catenin, other Wnt pathway components have been localized to the centrosomes23 and regulate centrosome functions. Axin1 is involved in MT nucleation,24 whereas Axin2 regulates centrosome cohesion.12 Moreover, APC and Dishevelled regulate the attachment and orientation of the mitotic spindle in which Wnt receptors Frizzled and LRP6 cooperate.25 Consequently, APC mutations lead to chromosome missegregation.26

β-catenin phosphorylated in Ser33/Thr47 was previously shown to accumulate at the centrosome, coordinating spindle dynamics8 and centrosome cohesion.12 Thus, Wnt or GSK3-β inhibitors would promote centrosome separation by maintaining β-catenin dephosphorylated at Ser/Thr.12 LiCl, a GSK3-β inhibitor, produces monoastral arrays, suggesting that it may affect MT arrays by decreasing PThr/Thr β-catenin. Nek2 directly phosphorylates β-catenin at Ser33/Thr47, thereby inhibiting β-catenin degradation and resulting in its accumulation at centrosomes.13 Importantly, PThr/Thr β-catenin is regulated through the cell cycle, peaks at G2/M [14] and remains at centrosomes during mitosis.8,13 Here, we described the novel centrosomal localization of PTyr142 β-catenin in different cell types, a form of β-catenin previously reported to be involved in cell migration, that localizes to the nucleus and regulates gene transcription.5,6 Interestingly, in contrast to centrosomal PThr/Thr β-catenin, PTyr142 β-catenin is undetectable in mitotic centrosomes. This finding suggests that a decline of centrosomal PThr142 β-catenin levels may be needed for centrosomal separation and/or mitotic progression. Several phosphatases (including Cdc25, PP1α, or PP2A) that control mitotic progression27–30 could selectively dephosphorylate PTyr142 β-catenin at the onset of mitosis.

PTyr142 β-catenin could associate to the centrosome in the phosphorylated state or be induced by a centrosomal kinase. Looking for putative Tyr kinases that could phosphorylate centrosomal PTyr142 β-catenin, we identified Syk as a putative candidate. Syk is a Tyr kinase localizing to the centrosome.15,31 Interestingly, Syk is persistent in interphase centrosomes but its levels drop in mitosis due to degradation by the proteasome system.15 We therefore addressed whether centrosomal Syk could regulate PTyr142 β-catenin by two approaches: overexpression of Syk active or inactive mutants and by treatment with the Syk inhibitor, Pic.19,20 Whereas cells overexpressing Tyr130Glu active Syk showed co-localization between active Syk and PTyr142 β-catenin at centrosomes, cells expressing Lys402Arg inactive Syk still displayed centrosomal PTyr142 β-catenin (results not shown). We reasoned that endogenous Syk could still be phosphorylating β-catenin upon expression of exogenous inactive Syk. Therefore, we investigated whether centrosomal PTyr142 β-catenin was affected by Pic. Cells treated with the Syk inhibitor displayed decreased centrosomal PTyr142 β-catenin, suggesting that Syk regulates its centrosomal levels. These findings are consistent with the loss of both Syk and PTyr142 β-catenin from centrosomes in mitosis. Moreover, Pic-treated cells showed a tendency to increase centrosome separation. Our observations suggest that PTyr142 β-catenin could contribute to the maintenance of centrosome cohesion, ensuring the interphase centrosomes to function as a single MT-organizing center. Upon mitotic entry, the absence of PTyr142 β-catenin and Syk at the centrosomes would allow their separation, correct bipolar spindle formation and chromosome segregation. Conversely, the Nek2 kinase phosphorylates β-catenin on Ser/Thr residues, stabilizes it at mitotic centrosomes and stimulates centrosome disjunction and splitting.13 The mechanisms behind these antagonistic roles of differently phosphorylated β-catenin forms and their balance remain to be unveiled by further in-depth functional studies.

Taken together, our results identify a novel β-catenin form that positively controls centrosome cohesion and point towards Syk as the kinase regulating β-catenin phosphorylation at the centrosomes. This study warrants future investigations on the functional implications of the distinct subcellular β-catenin pools in the centrosome cycle and cell division. The fine-tuned control of centrosome cohesion and separation are crucial for centrosome function throughout the cell cycle. Their aberrant regulation may lead to aneuploid defects characteristic of cancer cells.

ACKNOWLEDGMENTS

This work was funded by grants from Instituto de Salud Carlos III/ Feder/“Una manera de hacer Europa” (PI08/0790 and PI13/01980 to JH). DB was a recipient of FI-Agaur.
fellowship. MN, MCS, and AV were UdL predoctoral fellows. MN held an IRBLleida/Diputació de Lleida fellowship.

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How to cite this article: Bhardwaj D, Nager M, Visa A, et al. Phosphorylated Tyr142 beta-catenin localizes to centrosomes and is regulated by Syk. J Cell Biochem. 2018;119:3632–3640. https://doi.org/10.1002/jcb.26571