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

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ABSTRACT

β-catenin is a central component of adherent junctions and a key effector of canonical Wnt signalling, 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.

Short title: Centrosomal PTyr142 β-catenin

Keywords: β-catenin, centrosome, syk, glioblastoma, piceatannol
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 [Nigg et al., 2002; Gonczy et al., 2015].

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 signalling 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 signalling pathway, β-catenin acts as transcriptional co-regulator together with transcription factors of T-cell
Factor/Lymphoid Enhacer Factor families. Briefly, when signalling 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 [Brembeck et al., 2004; Heuberger et al., 2010], which is regulated by Met and Hepatocyte Growth Factor signalling [David et al., 2008; Nager et al., 2015] and other Tyr kinases [Piedra et al., 2003]. P(Tyr142 β-catenin therefore dissociates from the adhesion complex, localizes to the nucleus and promotes cell migration and axon growth in neurons [David et al., 2008; Nager et al., 2015].

While the classical roles of β-catenin both in cell adhesion and Wnt signalling 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 [Bahmanyar et al., 2008; Bahmanyar et al., 2010; Huang et al., 2007; Kaplan et al., 2004; Hadjihannas et al., 2010]. 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 [Bahmanyar et al., 2008; Bahmanyar et al., 2010; Hadjihannas et al., 2010].

β-catenin phosphorylated in Ser33/Ser37/Thr41 (PSer/Thr β-catenin; tagged for degradation when Wnt signalling is off) also accumulates in centrosomes [Huang et al., 2007], where it remains stable during mitosis [Mbom et al., 2014]. Expression of phospho-mimetic β-catenin mutants (Ser33/Ser37/Thr41 mutated to Glu) results in
multiple centrosomes and aberrant MT arrays [Huang et al., 2007], 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 signalling [Hadjihannas et al., 2012]. Furthermore, centrosomal PSer/Thr β-catenin regulates centrosome cohesion [Hadjihannas et al., 2010]. Consistent with this, Wnt signalling, GSK3 inhibitors, stabilized β-catenin or Axin2 knockdown promote centrosome separation [Hadjihannas et al., 2010].

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 [Zyss et al., 2005]. Furthermore, we show that Syk phosphorylates β-catenin at Tyr and that centrosome PTyr142 β-catenin levels decrease upon exposure to the Syk inhibitor piceatannol (Pic). Thus, we identify a PTyr142 β-catenin centrosomal pool regulated by Syk that, different to PSer/Thr β-catenin [Huang et al., 2007], vanishes from the centrosome in mitosis.
MATERIALS AND METHODS

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 Ethic 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 (EMFIs) 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 [Etienne-Manneville, 2006]. 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 1g/l glucose, 2 mM glutamine, 20 U/ml P/S and 10% FBS).

CELL TRANSFECTION

For knockdown using siRNA, RNAimax Lipofectamine was used. Briefly, 25 μl of Optimem containing siRNA sequences (80 nM; Sigma; 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 6h. Then media was changed for complete media (MEM including FBS).

ANTIBODIES

Anti-PTyr142 β-catenin antibodies were purchased from Abnova; anti-β-catenin from BD Bioscience or Cell Signalling (N-terminus), anti-γ-tubulin and anti-α-tubulin from Sigma-Merck, anti-Syk from Santa-Cruz Biotechnology and anti-PTyr (4G10) from Upstate Biotechnology.

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 6h. Pervanadate was prepared as previously described [David et al., 2008].

Mitosis arrest was carried out on cells plated on PDL-coated coverslips in complete media. Cells were deprived of serum for 20h and then grown in complete media for the 16-20h 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 (10x, 0.3 numerical aperture (NA); 20x, 0.4 NA; 32x, 0.4 NA) equipped with epifluorescence optics and a camera (Olympus OM-4 Ti) or using a
FluoViewTM FV1000 Confocal Microscope (60x). 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).

CENTROSONE ISOLATION

Centrosomal isolation was performed as described [Hsu et al., 1998]. Exponentially growing (8 p100 plates) U251MG cells were treated with Nocodazole (0.2 μM) and Cytochalasin D (1 μg/ml), 1h 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 (5,500 rpm, 10 min) and the supernatant filtered (70 μm). 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 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). Fractions were collected.
from the top: 500μl 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 10mM 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.

**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) [David et al., 2008]. Immunoprecipitated complexes were dissociated from the beads in loading buffer without β-Me.

**IN VITRO KINASE ASSAY**

GST–β-catenin recombinant proteins [Roura et al., 1999] were produced in BL21 bacteria. GST–β-catenin WT or Y142F proteins (1 μg; 8 pmols) were phosphorylated by recombinant Syk kinase (Precisio™, Sigma) 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.

**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 (1h, 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.
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 [Hsu et al., 1998]. Fractions 2 and 3, corresponding to ~50-60% sucrose and identified by a peak of γ-tubulin, were considered as the centrosome-enriched fractions [Hsu et al., 1998] (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-fractioned with γ-tubulin and β-catenin (Figure 2A), consistent with its centrosome localization [Zyss et al., 2005]. 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 20h followed by a period of 16-20h 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 [Zyss et al., 2005] and phosphorylates cell adhesion proteins (E-cadherin and α-catenin) [Larive et al., 2009]. Immunostaining 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 [Oliver et al., 1994] (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 vs. control cells (by 40-50% in U251MG and U87MG cells, respectively, according to fluorescence intensity measurements; Figure 4B and 4C). Centrosomal PTyr142 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 activator [Larive et al., 2009] (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 PTyr142 β-catenin may be involved in centrosomal cohesion/separation. Together, these results indicate that Syk phosphorylates centrosomal β-catenin in Tyr142.
DISCUSSION

Although β-catenin’s best studied roles include the transcriptional regulation of Wnt targets and cell adhesive functions [Valenta et al., 2012], the association of β-catenin with the centrosome has been known for years [Kaplan et al., 2004], when early studies showed its contribution to mitotic spindle formation and centrosome separation [Bahmanyar et al., 2008; Kaplan et al., 2004]. Mitogenic Wnt/β-catenin signalling regulates cell proliferation, and β-catenin and Wnt pathway components located to the centrosomes assist mitosis [Niehrs and Acebron, 2012]. Indeed, in addition to β-catenin, other Wnt pathway components have been localized to the centrosomes [Mbom et al., 2013] and regulate centrosome functions. Axin1 is involved in MT nucleation [Fumoto et al., 2009], whereas Axin2 regulates centrosome cohesion [Hadjihannas et al., 2010]. Moreover, APC and Dishevelled regulate the attachment and orientation of the mitotic spindle in which Wnt receptors Frizzled and LRP6 cooperate [Kikuchi et al., 2010]. Consequently, APC mutations lead to chromosome missegregation [Fodde et al., 2001].

β-catenin phosphorylated in Ser33/37/Thr47 was previously shown to accumulate at the centrosome, coordinating spindle dynamics [Huang et al., 2007] and centrosome cohesion [Hadjihannas et al., 2010]. Thus, Wnt or GSK3-β inhibitors would promote centrosome separation by maintaining β-catenin dephosphorylated at Ser/Thr [Hadjihannas et al., 2010]. LiCl, a GSK3-β inhibitor, produces monoastral arrays, suggesting that it may affect MT arrays by decreasing PSer/Thr β-catenin. Nek2 directly phosphorylates β-catenin at Ser33/37/Thr47, thereby inhibiting β-catenin degradation and resulting in its accumulation at centrosomes [Mbom et al., 2014]. Importantly, PSer/Thr β-catenin is regulated through the cell cycle, peaks at G2/M [14] and remains at centrosomes during mitosis [Huang et al., 2007; Mbom et al., 2014]. 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 [David et al., 2008; Nager et al., 2015]. Interestingly, in contrast to centrosomal PSer/Thr β-catenin, PTyr142 β-catenin is undetectable in mitotic centrosomes. This finding suggests that a decline of centrosomal PTyr142 β-catenin levels may be needed for centrosomal separation and/or mitotic progression. Several phosphatases (including Cdc25, PP1α or PP2A) that control mitotic progression [Meraldi et al., 2001; Boutros et al., 2007; Schlaitz et al., 2007; Bonnet et al., 2008] 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 [Zyss et al., 2005; Fargier et al., 2013]. Interestingly, Syk is persistent in interphase centrosomes but its levels drop in mitosis due to degradation by the proteasome system [Zyss et al., 2005]. 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 [Oliver et al., 1994; Larive et al., 2009]. 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 [Mbom et al., 2014]. 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.
ACKNOWLEDGEMENTS

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**FIGURE LEGENDS**

**Fig. 1. PTyr142 β-catenin is found in centrosomes.** A) Immunostaining for PTyr142 β-catenin and the centosome marker γ-tubulin in rat striatal astrocytes, U251MG and U87MG GBM cells and mouse embryonic fibroblasts (EMFIs). 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 [Nager et al., 2015]. 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.

**Fig. 2. PTyr142 β-catenin co-fractionates with γ-tubulin.** A) PTyr142 β-catenin co-fractionates in centrosome-enriched fractions isolated from U251MG cells. Gradient fractions (0-8) were blotted as indicated. Anti-γ-tubulin antibodies identify a peak of centrosome-enriched fractions (fractions 2 and 3, corresponding to 50-60% sucrose), in which PTyr142 β-catenin, total β-catenin and Syk co-fractionate. Samples of the 1st and 2nd total lysates from the sub-fractionation procedure are shown on the left. B) β-catenin immunoprecipitation (IP) from U87MG cells shows γ-tubulin associated to β-catenin immunoprecipitates containing PTyr142 β-catenin (lys, lysate).

**Fig. 3. PTyr142 β-catenin disappears from centrosomes during mitosis.** Immunostaining for PTyr142 β-catenin (red) and α-tubulin (green) in U251MG cells synchronized by serum deprivation. Mitotic figures are boxed and enlarged. Immunostaining for α-tubulin allowed the visualization of MTs and, together with Hoechst (blue), helped identifying the mitotic stage. Note that interphase and daughter...
cells (after cytokinesis) show centrosomal PTyr142 β-catenin immunostaining (arrows). Images correspond to confocal sections. Bar= 15 µm.

**Fig. 4. Piceatannol (Pic) decreases PTyr142 β-catenin centrosomal levels.** A) Syk recombinant kinase assay using WT or Y142F β-catenin proteins. Anti-PTyr antibodies were used to detect both GST-β-catenin (120 kDa) and GST-Syk (100 kDa). PTyr phosphorylation of WT β-catenin increased upon addition of ATP (indicated by +) compared to the first lane (- ATP), and it was reduced by Staurosporine (stau) and Pic in the presence of ATP. These results correlate with the PTyr phosphorylation levels of Syk (some kinase activity is detected in the absence of ATP, first lane). PTyr phosphorylation of Y142F β-catenin was reduced compared to that of WT β-catenin, showing similar levels with or without Pic. β-catenin WB shows similar input of WT and Y142F proteins. B) U251MG cells (control or treated with Pic 200 µM) immunostained using anti-PTyr142 β-catenin and anti-γ-tubulin antibodies. PTyr142 β-catenin (red) and γ-tubulin (green) immunostainings co-localize at centrosomes in control cells, whereas in Pic-treated cells centrosomal PTyr142 β-catenin decreases. Arrows point at positive PTyr142 β-catenin centrosomes. Bar= 15 µm. C) Quantification of centrosomal PTyr142 β-catenin intensities (A.U.) demonstrates its decreased levels upon Pic treatment (***, P< 0,001) in U251MG and U87MG cells. N.S. indicates non-significant differences. D) Plot presenting the % of U251MG cells (control or treated with Pic) displaying one apparent centrosome, two closely adjacent centrosomes or two separated centrosomes per cell, as observed by γ-tubulin immunostaining (* P<0,05).
REFERENCES


Bhardwaj et al. Fig. 2

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PTyr142 β-cat
β-cat
γ-tubulin
Syk

Centrosomal fractions

B

Lys  IP

PTyr142 β-cat
γ-tubulin
β-cat
Bhardwaj et al., Fig. 4

A

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WB: PTyr

Control                 Piceatannol

centrosomal PTyr142 β-catenin (A.U.)

B

PTyr142 β-cat        γ-tubulin       merge

Control

Piceatannol

Scale bar: 10 μm

C

U251MG

U87MG

D

percentage of cells

Control    Piceatannol

1 centr.  2 adjacent  2 separated