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PP2A phosphatase suppresses function of the mesenchymal invasion regulator NEDD9

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ABSTRACT

The mesenchymal mode of cancer cell invasion characterized by active adhesion turnover and a polarized actin cytoskeleton, is critically regulated by the adaptor protein NEDD9/HEF1/Cas-L. While it is known that NEDD9 is subject to extensive phosphorylation modification, the molecules that determine NEDD9 phosphorylation to stimulate adhesion turnover and mesenchymal cell morphologies are currently unknown. Earlier studies have suggested that the serine/threonine phosphatase PP2A regulates interconversion between a low molecular mass NEDD9 phosphoform and higher molecular mass phosphoforms. However, previous studies have used chemical inhibitors to block PP2A activity. In the present study we therefore aimed to specifically inhibit PP2A activity via siRNA and dominant negative approaches to investigate the effect of PP2A on interconversion between 115 kDa and 105 kDa NEDD9 and determine the functional consequence of PP2A activity for NEDD9 function. Strikingly, we find that while the phosphatase inhibitor Calyculin A indeed abrogates detachment-induced dephosphorylation of the 115 kDa NEDD9 phosphoform, PP2A depletion does not inhibit 115 kDa to 105 kDa interconversion. Our data suggest instead that PP2A targets discrete NEDD9 phosphorylation modifications separate to the events that mediate interconversion between the two forms. Functionally, PP2A depletion increases NEDD9 mediated cell spreading and mutation of S369 in the serine-rich region of NEDD9 to aspartate mimics this effect. Importantly, mutation of S369 to alanine abrogates the ability of dominant negative PP2A to increase NEDD9-mediated cell spreading. Collectively, our data reveal that the tumour suppressor PP2A may act via S369 to regulated NEDD9-mediated cell spreading.
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1. Introduction

Individually invading cancer cells may switch between mesenchymal and amoeboid invasion modes, depending on the composition of the surrounding matrix [1,2]. The mesenchymal invasion mode is characterized by a polarized actin cytoskeleton and depends on the regulated formation and disassembly of integrin-based adhesions to the surrounding extra-cellular matrix [3]. Recent advances have revealed that the adhesion docking protein NEDD9/HEF1/Cas-L is part of a signalling pathway that stimulates mesenchymal invasion and antagonizes amoeboid invasion [4]. Elevated expression of NEDD9 has been implicated as a metastasis promoter in a variety of tumour types [5]. NEDD9 contains multiple consensus serine, threonine and tyrosine phosphorylation motifs and extensive phosphorylation modification of the protein suggests that this is key to NEDD9 function [6]. Potential regulators of NEDD9 phosphorylation status are the PP2A phosphatases [7]. This enzyme family displays tumour suppressor activity [8] and regulates a variety of cellular signalling pathways; however the consequence of PP2A activity for NEDD9 adhesion-mediated functions has not been reported.

NEDD9 is an adhesion docking protein that forms part of a signalling hub at integrin-mediated adhesion sites [6]. Lacking enzymatic activity, NEDD9 regulates the formation of signalling complexes via its protein–protein interaction domains: a Src Homology 3 (SH3) domain; followed by an SH2-binding domain that contains at least 17 tyrosine residues that can be phosphorylated *in vivo* (Phosphosite database [9]); a serine-rich region encompassing 27 serine residues and; a highly-conserved C-terminal domain [10]. Phosphorylation of serines, threonines and tyrosines in the NEDD9 sequence modulates NEDD9 interaction with down-stream signalling partners and thus regulates NEDD9's signalling function.

NEDD9 is frequently reported as a doublet of ~105 kDa and 115 kDa molecular mass by Western blot and more recently a third intermediary phosphoform was reported [11]. The relative expression of the NEDD9 phosphoforms differs between cell lines and tissues

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[12]. 105 kDa NEDD9 is phosphorylated on multiple tyrosine residues and serine residues, while the 115 kDa form is additionally phosphorylated on threonine [13–15]. 105 kDa NEDD9 interconverts to 115 kDa NEDD9 in response to integrin-mediated adhesion [14,16]. Following treatments that disrupt either the actin cytoskeleton or adhesion, 115 kDa NEDD9 collapses to form 105 kDa NEDD9. This can be abrogated by treatment with Okadaic acid and Calyculin A [14,16,17}. Based on the data obtained using Okadiac acid and Calyculin A—two natural products with PP2A inhibitory activity—a model has been proposed whereby cell detachment leads to PP2A activation, which is then proposed to catalyze the interconversion from 115 kDa to 105 kDa NEDD9 [7]. Moreover, a recent study demonstrated that mutation of S369 in the NEDD9 serine-rich region specifically abrogated Okadaic acid-stimulated interconversion between 105 kDa and 115 kDa NEDD9 [11]. This suggests that PP2A may target NEDD9 S369. However, both Okadaic acid and Calyculin A inhibit phosphatases in addition to PP2A [14,18]. Thus to specifically demonstrate the role of PP2A in the interconversion between 115 kDa and 105 kDa NEDD9, approaches that directly target PP2A are required.

The PP2A holoenzyme exists as a tri-molecular complex of regulatory (B), catalytic (C) and scaffolding (A) subunits and multiple isoforms of each subunit provides alternative substrate specificities and sub-cellular distributions. An extensive body of work now provides significant support for the notion that PP2A phosphatases have tumour suppressor activity and it is emerging that one of the functions of this protein may be to suppress cell migration. Inhibition of PP2A activity via mutation in a regulatory B subunit stimulated rapid and extensive cell spreading and the switch to a polarized cytoskeleton characteristic of the mesenchymal morphology [19], while siRNA-mediated targeting of the PP2A catalytic subunit enhanced cell migration [20]. Notably, NEDD9 overexpression also promotes cell spreading [12,16,21,22] and leads to a mesenchymal pattern of actin rearrangement [21]. This switch to mesenchymal invasion is characterized by a polarized actin cytoskeleton in which a short mesh work of actin filaments is localized to the protruding edge and behind the leading edge discrete actin filament populations are arrayed perpendicular (dorsal arcs) and parallel (transverse arcs) to the leading edge [3]. Thus inhibition of PP2A activity and increased NEDD9 expression stimulate similar cellular phenotypes, suggesting the possibility that PP2A may suppress NEDD9-mediated spreading and inhibit the switch to a mesenchymal phenotype.

The aim of the present study was to compare the effects of Calyculin A treatment versus PP2A knock-down on the interconversion between 115 kDa and 105 kDa NEDD9 and investigate the functional consequence of PP2A activity for NEDD9 function. Our data suggest that PP2A does not regulate the interconversion between 115 kDa and 105 kDa NEDD9 but instead targets discrete NEDD9 phosphorylation modifications separate to those that mediate interconversion. We show that PP2A suppresses NEDD9 mediated cell spreading and demonstrate a requirement for NEDD9 S369 in this process.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal antibodies to NEDD9 (clone 2G9) were from ImmuQuest (Cleveland, UK), monoclonal anti-tubulin and purified mouse IgG were purchased from Sigma (Missouri, USA) and phospho-specific anti-PP2A-C Y307 monoclonal antibodies were from Epitomics (California, USA). Antibodies against PP2A-C (clone 1D6) were from Upstate (California, USA). Secondary HRP-conjugated anti-mouse and anti-rabbit antibodies were from Amersham (New Jersey, USA) and Biorad (California, USA) and 4′,6-diamidino-2-phenylindole (DAPI) was from Sigma. Cy3-tagged anti-phalloidin was purchased from Sigma and reconstituted in methanol. Calyculin (Chemicon, California, USA) was resuspended in DMSO and MG132 was purchased from Calbiochem (CA, USA). SiGENOME pooled siRNAs directed against the PP2A subunit and non-targeting siRNA pool #1 were purchased from Dharmacon (Colorado, USA) and reconstituted in RNAse free water.

2.2. Creation of GST-fusion peptides and expression plasmids with site-specific mutations

Expression plasmids encoding NEDD9 fused with GFP have been previously described ([16]. The GST.SRR expression construct was prepared by inserting cDNA coding for NEDD9 amino acids 351–653 into EcoR1–XhoI cloning sites of the pET-41a(+) vector (Novagen). Site specific mutation of Serine 369 to either alanine (S369A) or aspartate (S369D) was achieved using a PCR-based mutagenesis as previously described [23] using GFP.NEDD9 as the template. Primers for mutagenesis were as follows: S369A (forward: 5′GGATGGGATCAACCGATTGGCTTTCTCCAGTACAGGCAGCAC3′, reverse: 5′GTGCTGCCTGTACTGGAGAAAGCCAATCGGTTGATCCCATCC3′) and S369D (forward: 5′GGATGGGATCAACCGATTGGATTTCTCCAGTACAGGCAGCAC3′, reverse 5′GTGCTGCCTGTACTGGAGAAATCCAATCGGTTGATCCCATCC3′). Details of the dominant negative PP2A catalytic subunit (PP2Ac-L199P) have been previously reported [24].

2.3. Cell culture

MCF-7 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen, Victoria, Australia) supplemented with 10% FBS (Invitrogen) at 37 °C with 5% CO_2. For all experiments, cells were cultured in phenol-red free DMEM plus 10% charcoal treated FBS supplemented with 10 nM estradiol for at least 7 days prior to the experiment. Development of the tetracycline-regulatable MCF-7 line was previously described [21]. NEDD9 expression was induced by culturing cells in the absence of tetracycline for 16–24 h.

2.4. RNA interference

For siRNA knock-down cells (7×10^5) were plated in 35 mm tissue culture dishes and incubated overnight. Transfections were performed using RNAiMax (Invitrogen) according to the manufacturer's instructions. Briefly, RNAiMaxor and 3 pmol of siRNA were incubated to form complexes in Opti-Mem for 20 min and cells then incubated with RNAiMaxor-RNA solution for 6 h. Transfections were repeated in the same cells 24 h later to achieve robust knock-down and cells were collected for analysis 72 h post -initial transfection.

2.5. Phosphorylation kinetics

Cell quiescence was achieved as described previously [7]. Briefly, 24 h post -final siRNA transfection, culture media was changed to serum-free DMEM with 0.1% heat inactivated bovine serum albumin (hiBSA). Cells were then rinsed with PBS and detached by incubation with 3 mM EDTA at 37 °C for 15 min. Following resuspension in hiBSA, cells were held in suspension by plating into bacterial Petri dishes for the required time. The zero time point represents cells following detachment with 3 mM EDTA prior to resuspension in hiBSA. For all time points floating cells were collected, washed in PBS and extracted in PTY lysis buffer as normal.

2.6. Co-immunoprecipitation and GST pull-downs

For co-immunoprecipitation analysis, cells were washed twice with ice-cold PBS and incubated in lysis buffer (10 mM Tris–HCl pH 7.5, 5 mM EDTA pH 8.0, 50 mM sodium fluoride, 10 mM dithiothreitol, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1% nonidet P-40 (NP-40), 0.05% SDS, 0.25% sodium deoxycholate, 10 μg/ml leupeptin, 10 μg/ml aprotinin) at 4 °C for 30 min. Lysates

were briefly centrifuged and supernatants collected. Protein A-Sepharose (Sigma) was prepared by swelling beads in 10 mM HEPES buffer, pH 7.2 for 30 min on ice then washing in PBS and resuspending beads in an equal volume of lysis buffer. Lysates were pre-cleared with protein A-sepharose for 1 h at 4 °C then collected by centrifugation (10,000×*g*) and resulting supernatants incubated with anti-NEDD9, PP2A-C or isotype-matched, mouse IgG antibodies at 4 °C for 2 h. After the addition of protein A-Sepharose slurry, incubations were continued overnight at 4 °C. The resin was collected and washed five times with 0.5% NP-40 in phosphate-buffered saline then proteins dissociated from the beads by boiling.

For GST pull-downs, bacterial cells were washed with ice-cold PBS and incubated in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM sodium fluoride, 2% nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.36 mM sodium orthovanadate, Protease inhibitor cocktail (Sigma)) at 4 °C for 10 min. Lysates were briefly centrifuged and supernatants collected. The lysate was pre-cleared of endogenous GSTs and non-specifically binding proteins present by incubating with Glutathione Sepharose 4B (GE Healthcare) three times for 30 min. Pre-cleared lysate (250 µl) was incubated with 20 µl of 1 mg/ml GSH-GST.SRR beads or GSH-recombinant GST (control) beads at 4 °C for 1 h. Beads were transferred to a Micro Bio-spin column (BioRad) and washed six times with TBS and four times with 20 mM Tris, pH 7.4. Proteins bound to the beads were eluted in 1× SDS sample loading buffer at 85 °C.

2.7. SDS-PAGE and Western blotting

Where indicated, cells were pre-treated with Calyculin A for 4 h. As the addition of Calyculin A stimulated cell rounding, in all Calyculin treated experiments floating cells were collected and extracted for analysis. Protein extractions, equalisation and immunoblotting were performed as previously described [25]. Proteins were separated by electrophoresis in 6% acrylamide SDS-polyacrylamide gels to detect NEDD9 and 12% to detect PP2A-C.

2.8. Imaging and image analysis

Cells grown on coverslips were fixed in 4% paraformaldehyde for 10 min, permeabilised in wash solution (0.2% triton X-100 in 0.5% BSA) for 5 min then incubated with Cy3 labelled phalloidin diluted 1/1000 in wash solution for 1 h at room temperature. Cells were then counterstained with DAPI (diluted 1/10,000) for 15 min and coverslips mounted onto microscope slides with FluorSave (Calbiochem, San-Diego, USA). Fluorescent imaging was performed using an Olympus IX81 inverted microscope (Olympus, Pennsylvania, USA) and images captured by using an ORCA ERG cooled CCD camera (Hamamatsu/SDR Clinical Technology). Final micrograph images and gray level adjustments were prepared in Adobe Photoshop. Cell measurements were carried out using Metamorph V6.3 software and final data analysis in X-cel. Densitometric analysis of protein detection on Western blots was achieved using ImageJ 1.34S software (NIH, USA).

3. Results

3.1. PP2A does not regulate the interconversion of 115 kDa to 105 kDa NEDD9

Detachment from the sub-stratum and treatments that disassemble the actin cytoskeleton all lead to the loss of 115 kDa NEDD9 [14,16,17] and blockade of this effect with either Calyculin A or Okadaic acid has led to the suggestion this is mediated by PP2A activity [7]. That is, PP2A has been suggested to mediate detachment-induced dephosphorylation of 115 kDa NEDD9 to produce 105 kDa NEDD9. Consistent with previous observations, treatment with either Calyculin A (Fig. 1A) or Okadaic acid (data not shown) robustly inhibited suspension-induced

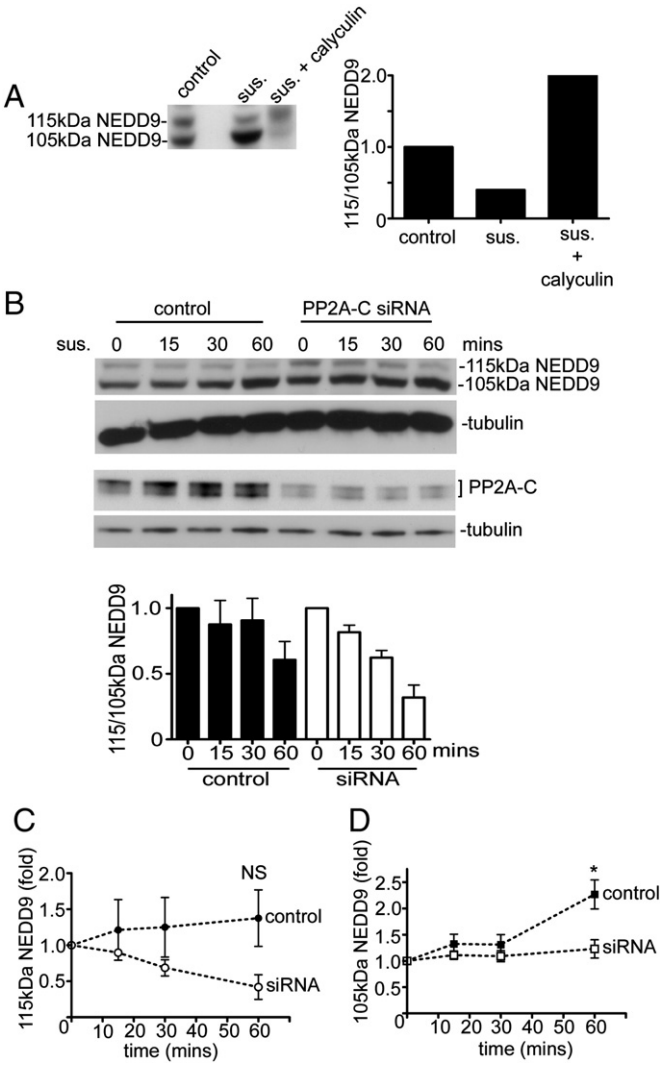

Fig. 1. PP2A does not regulate 105 kDa to 115 kDa NEDD9 interconversion. A. Western blot detection of NEDD9 expression in lysates from attached MCF-7 cells (control), cells placed in suspension (sus.) or pre-incubated with 10 nM calyculin for 4 h and then placed in suspension. The histogram shows representative data of the ratio of 115 kDa/105 kDa NEDD9 for each condition, relative to the control sample. B. MCF-7 cells transfected with control siRNA (control) or siRNA targeting PP2A-C, were placed in suspension (sus.) and incubated for the indicated times (min). The histogram underneath shows the ratio of 115 kDa/105 kDa NEDD9 with time, expressed relative to the level at '0'. The heavy signal of the tubulin loading control for the NEDD9 blot reflects the quantity of total protein (75 µg) required in order to visualize NEDD9; tubulin was not used to normalize protein expression levels. C and D. 115 kDa NEDD9 and 105 kDa expression, respectively, in suspension cultures relative to the zero time point. Black points show data for cells treated with control siRNA and white points represent data for cells treated with PP2A-C siRNA. All experiments were repeated in triplicate on separate days and the average of three experiments is shown. Error bars show the SEM. Statistical analysis was performed using two-way ANOVA with Bonferroni post-tests, $^{*}p<0.001$, NS = not significant.

accumulation of 105 kDa NEDD9. Both 105 kDa and 115 kDa NEDD9 appear to have reduced electrophoretic mobility in the presence of Calyculin, suggesting that there may be multiple phospho-intermediates of each form. Moreover, the total levels of NEDD9 are decreased in the presence of calyculin agreeing with earlier data reporting targeting of hyper-phosphorylated NEDD9 by the proteasome [7]. These data are in accord with earlier reports indicating that Calyculin A and Okadaic acid both block suspension-induced interconversion from 115 kDa NEDD9 to 105 kDa NEDD9.

We next used siRNA targeting the catalytic subunit of PP2A (PP2A-C) to establish the role of PP2A in the detachment-induced interconversion of 115 kDa and 105 kDa NEDD9. Knock-down of PP2A

routinely achieved >50% reduction in PP2A-C protein (Fig. 1B) and kinetic analysis of NEDD9 phosphoforms in suspended cells following PP2A-C knock-down was performed. Comparison of the effects of Calyculin A treatment and PP2A depletion revealed striking differences in NEDD9 regulation (compare Fig. 1A and B). There is little evidence of 115 kDa NEDD9 accumulation in suspended cells following PP2A knock-down (Fig. 1B). Moreover, the PP2A-C knock-down suspended cells retained prominent expression of 105 kDa NEDD9 (Fig. 1B), in direct contrast to effects seen with Calyculin A treatment. Analysis of the change in phosphoforms over the 60-minute time course revealed depletion of 115 kDa NEDD9 and no change in the levels of 105 kDa NEDD9 in suspended cells following PP2A depletion (Fig. 1C and D). The differences observed between the effects of Calyculin A and PP2A siRNA therefore suggest that unlike Calyculin A treatment, PP2A depletion does not block the suspension-induced conversion of 115 kDa to 105 kDa NEDD9. Given that NEDD9 is a highly phosphorylated protein that contains multiple serine residues, PP2A may therefore target phosphorylated residues of NEDD9 that are distinct from those that mediate interconversion between 105 kDa and 115 kDa NEDD9.

3.2. NEDD9 and PP2A interact via the NEDD9 SRR domain

To further investigate the potential interaction between NEDD9 and PP2A we next determined whether NEDD9 interacts with PP2A *in vivo*, by performing co-immunoprecipitations from cell lysates. This revealed that PP2A-C co-immunoprecipitates with anti-NEDD9 antibodies and conversely, that NEDD9 co-immunoprecipitates with anti-PP2A-C antibodies (Fig. 2A). Notably, both NEDD9 phosphoforms were observed to co-immunoprecipitate with PP2A and the ratio of co-immunoprecipitating 105 kDa to 115 kDa NEDD9 (that is, greater levels of 105 kDa NEDD9) is similar to the ratio of endogenous NEDD9 in the whole cell lysate (Fig. 2A(ii)). For additional confirmation that NEDD9 forms a complex with PP2A we performed GST-pull-downs. As it is not possible to express full length NEDD9 in bacteria, we instead used the NEDD9 serine-rich region (SRR) that has previously been suggested to have the same tertiary structure as the SRR domain of the highly related molecule p130Cas [26]. The high level of serine residues encompassed by the SRR suggested that PP2A may interact with NEDD9 via this domain, therefore we prepared expression constructs encoding the SRR domain fused to GST (Fig. 2B and C). Importantly, pull-downs with the GST.NEDD9.SRR from cell lysates successfully isolated the catalytic subunit of PP2A (Fig. 2D), therefore demonstrating that PP2A interacts with the SRR domain of NEDD9.

3.3. PP2A regulates NEDD9-mediated cell spreading

It is known that elevated NEDD9 expression stimulates cell spreading [12,21,27] and the adoption of a polarized actin cytoskeleton that characterizes the mesenchymal morphology [3,21]. Therefore, we next measured the effect of PP2A knock-down on NEDD9-mediated cell spreading and morphologies, following NEDD9 induction as previously described [21]. First, we examined the effect of PP2A-C knock-down on total NEDD9 phosphoforms in the adherent cells that had been induced to express NEDD9 (Fig. 3A and B). In the adherent cells, little difference was observed in NEDD9 phosphoforms subsequent to PP2A-C knock-down (Fig. 3B). By comparison,

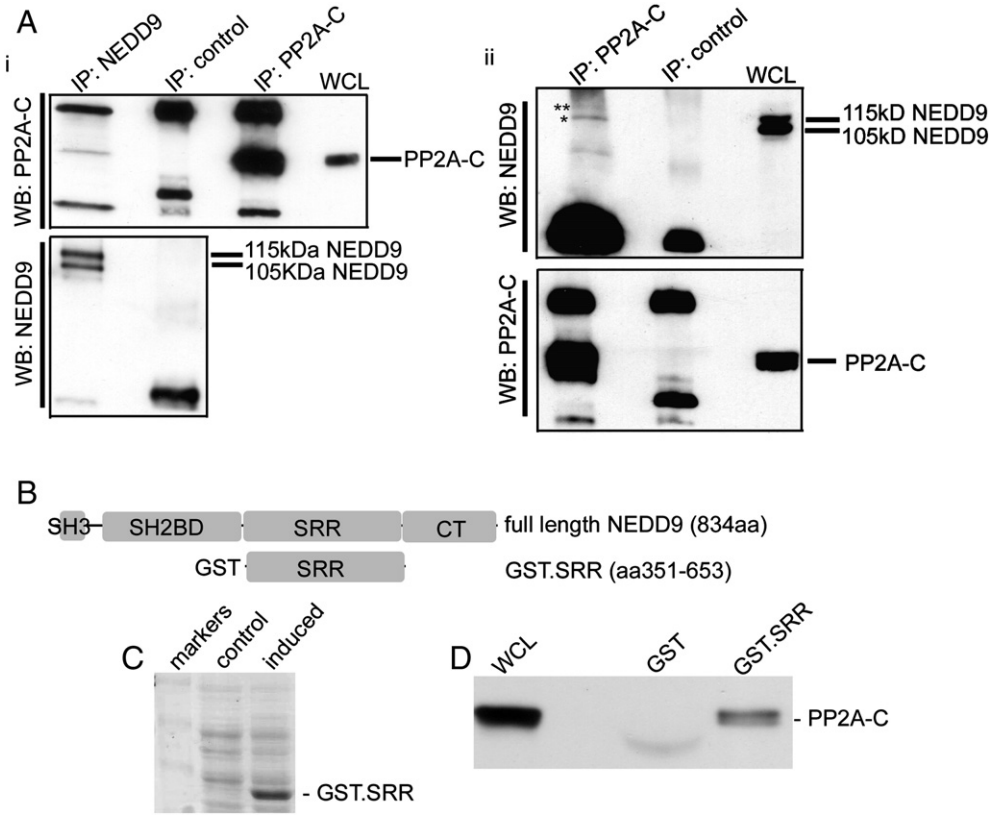

Fig. 2. NEDD9 SRR interacts with PP2A. A. Proteins were immunoprecipitated (IP) with the indicated antibodies (control = isotype-matched antibody control) from extracts of cells induced to express NEDD9. Western blots were probed with the indicated antibodies (WB) and whole cell lysates (WCL) included as controls. (i) demonstrates PP2A-C co-immunoprecipitating with anti-NEDD9 antibody complexes and (ii) shows NEDD9 co-immunoprecipitating with anti-PP2A-C complexes. *105 kDa NEDD9, **115 kDa NEDD9. B. Schematic representation of NEDD9 protein domains in full length NEDD9 (834 amino acids) (SH3: Src Homology 3, SH2BD: Src Homology 2 Binding Domain, SRR: Serine Rich Region, CT: C-terminus) and the GST.SRR fusion protein used in GST pull-down experiments. C. Coomassie stained gel showing the induction of GST.SRR. Protein extracts were prepared from bacteria transformed with the GST.SRR expression construct under control conditions (control) or following induction of the fusion protein (induced). D. Western blot detection of PP2A-C in whole cell lysates (WCL) and following pull-down with glutathione beads coupled to GST control and with the GST.SRR fusion protein.

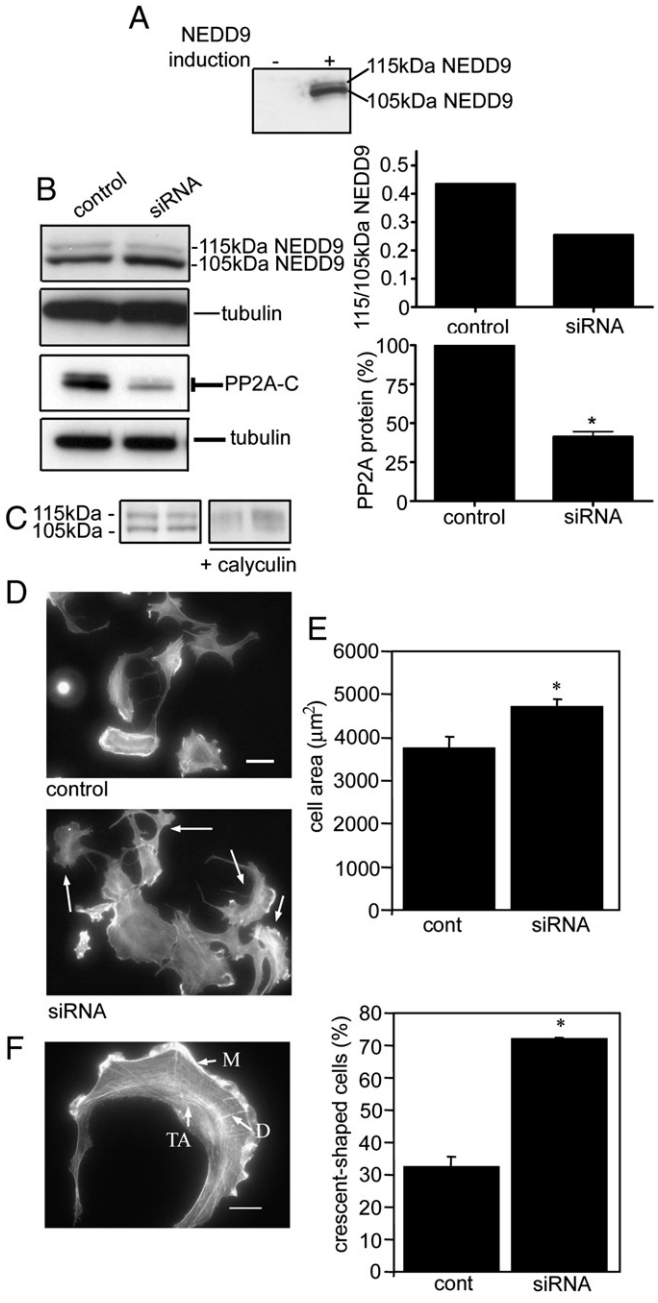

Fig. 3. PP2A-C knock-down promotes NEDD9-mediated cell spreading. A. Western blot confirming induction of NEDD9. B. NEDD9 and PP2A-C in protein extracts from the inducible NEDD9 expression cells transfected with siRNA targeting the PP2A catalytic subunit (PP2A-C) or control siRNA. Blots were probed with tubulin to confirm equal protein loading. Top histogram shows representative data of the ratio of 115 kDa NEDD9 to 105 kDa NEDD9 under the indicated conditions. The lower histogram shows the average % PP2A-C protein remaining following siRNA targeting, from 3 independent experiments. C. Western blot of lysates from adherent MCF-7 cells under control conditions or treated with Calyculin A. Duplicate samples are shown. D. Cells induced to express NEDD9 and transfected with control or PP2A-C targeted siRNA, fixed and probed with fluorescently-tagged phalloidin to detect filamentous actin. Arrows indicate typical crescent-shaped cells. 50 μm scale bar. E. Area of cells induced to express NEDD9 and transfected with control or PP2A-C targeted siRNA. Data represent the mean of 3 independent experiments (>45 cells measured per experiment). F. Higher magnification image (60× objective) of typical crescent-shaped cell from a culture expressing elevated NEDD9 and transfected with PP2A-C targeted siRNA. Fluorescently-tagged phalloidin detects the meshwork of short actin filaments in the leading edge (M), the transverse arcs (TA) arrayed parallel to the leading edge and the dorsal stress fibres (D) at right angles to the leading edge. 10 μm scale bar. Histogram indicates the percentage of crescent-shaped cells. Data represent the average of 3 independent experiments. Error bars represent the S.E.M. *$p<0.05$, Students *t*-test.

Calyculin A treatment of adherent MCF-7 cells causes a striking loss of 105 kDa NEDD9 and a smear of high molecular weight isoforms (Fig. 3C) similar to the pattern seen in suspended cells treated with Calyculin A (Fig. 1A). Remarkably, knock-down of PP2A-C combined with induced NEDD9 expression stimulated significantly increased cell spreading when compared with the induction of NEDD9 alone (Fig. 3D and E). Examination of cell morphologies following PP2A-C knock-down additionally revealed significantly increased crescent-shaped cells, typified by polarized distribution of actin filaments including an actin filament meshwork at the leading edge, transverse arcs parallel to the leading edge and dorsal stress fibres perpendicular to the leading edge (Fig. 3F). Thus, these data suggest that PP2A reduces NEDD9-mediated effects on cell spreading and mesenchymal cell morphology.

3.4. Increased cell spreading following PP2A depletion and elevated NEDD9 requires S369

Mutation of NEDD9 serine 369 to alanine abrogates the formation of higher molecular weight forms of NEDD9 in response to Okadaic acid treatment [11]. Agreeing with this, we find that the S369A mutation also blocks formation of 115 kDa induced by Calyculin A treatment (Fig. 4B). To next determine whether constitutive phosphorylation of S369 rescues the formation of 115 kDa NEDD9, we prepared a phospho-mimetic construct in which S369 was mutated to aspartate (S369D). However, no doublet of S369D mutant protein was observed, even after longer exposure times of the autoradiograph (Fig. 4B). Similarly, although treatment with Calyculin A stimulated increased 115 kDa GFP.NEDD9 expression relative to the 105 kDa form, S369D remained as a single lower molecular mass band (Fig. 4B). The lack of an S369D doublet does not appear to be due to proteasomal degradation of the upper 115 kDa form, as neither treatment with MG132 alone nor in combination with Calyculin A stimulated a prominent doublet of this mutant protein (Fig. 4B). Thus these data suggest that the phospho-mimetic mutation cannot rescue 115 kDa NEDD9 formation.

Importantly, both S369A and S369D were detected at focal adhesions, as seen for the wild-type NEDD9 GFP fusion protein (GFP.NEDD9) (Fig. 4C). To determine the consequence of the S369 point mutations for cell spreading, we measured cell area following transfection with each construct compared to empty vector (GFP) and wild-type NEDD9 (GFP.NEDD9) controls. This revealed that the S369A mutant protein increased the cell area to the same extent as seen for cells expressing wild-type GFP.NEDD9 (Fig. 4C). In contrast, the S369D mutant construct stimulated a striking further increase in cell area, resulting in cells with significantly larger area than either GFP.NEDD9 or S369A transfectants (Fig. 4C). Together, these data suggest that although phosphorylation of S369 is not absolutely required for NEDD-mediated cell spreading, it is sufficient to stimulate increased cell spreading.

To investigate the relationship between S369 and PP2A activity, we next employed a PP2A dominant negative expression construct, which allowed us to examine combinations of PP2A inhibition together with NEDD9 expression constructs (Fig. 5A–C). Quantification of cell areas revealed that PP2A.DN alone promoted increased MCF-7 cell spreading (Fig. 5A and C). Agreeing with the earlier data showing that elevated NEDD9 expression and PP2A siRNA simulated significantly increased cell area (Fig. 3E), combined expression of PP2A.DN and GFP.NEDD9 resulted in cell areas that were significantly larger than cells transfected with either PP2A.DN or GFP.NEDD9 alone (Fig. 5C). Critically, this effect was lost when S369A was co-expressed with PP2A.DN, as there was no difference in area between cells expressing PP2A.DN and S369A either alone or in combination (Fig. 5C). This suggests that the increased cell area resulting from combined exogenous NEDD9 expression and decreased PP2A activity may be mediated by S369.

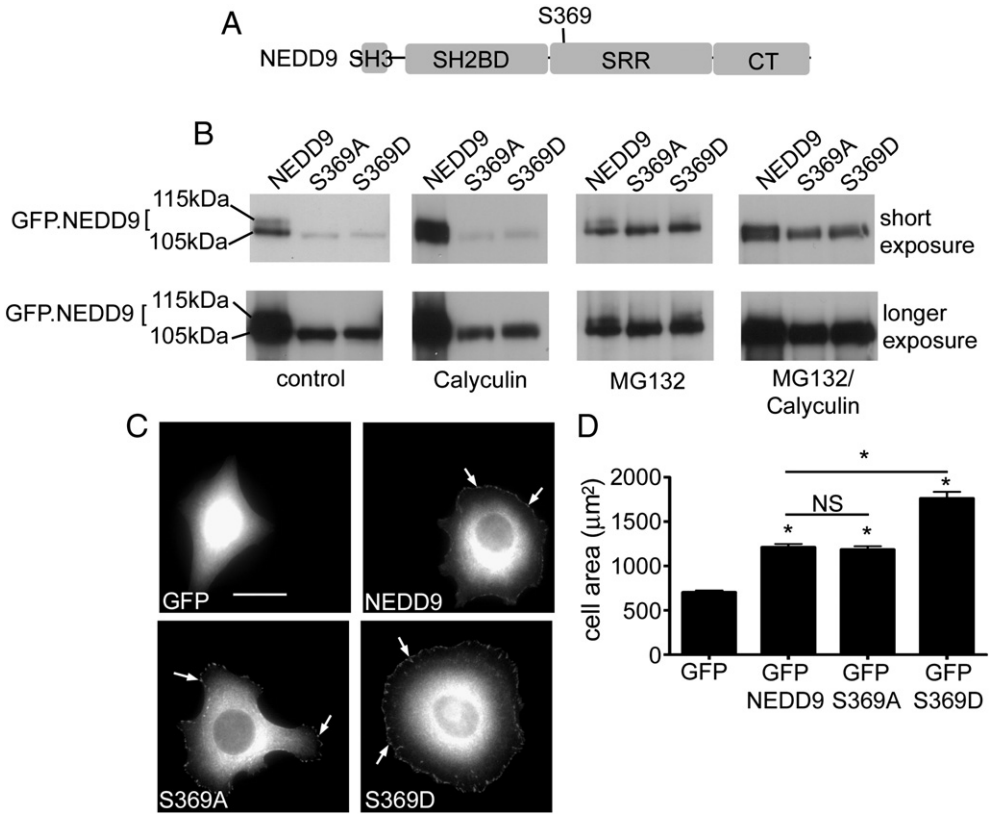

Fig. 4. Mutation of NEDD9 S369 recapitulates PP2A knock-down effects. A. Schematic representation of NEDD9 protein sequence indicating position of S369. B. Western blot analysis of MCF-7 cells transfected with the indicated expression constructs. Exogenous GFP-fused NEDD9 protein phosphoforms are indicated. Transfected cells were grown under control conditions, in the presence of Calyculin A or MG132 alone and in combination. Two different exposure times (short and longer) of autoradiographs are shown to aid in the identification of GFP.NEDD9 phosphoforms. Note that only the lanes with wild-type GFP.NEDD9 display the high molecular mass 115 kDa NEDD9 phosphoform. C. Images of MCF-7 cells transfected with the indicated expression constructs. Arrows point to GFP fusion proteins localized to focal adhesions. Scale bar 20 μm. D. Average area of GFP positive cells. Data are calculated from triplicate repeats on separate occasions. Values were compared using one-way ANOVA with Tukey's Multiple Comparison test, $^{*}p<0.05$, NS = not significant.

4. Discussion

The PP2A serine/threonine phosphatases regulate a variety of cellular signalling pathways and disruption of these pathways via loss of PP2A activity results in cellular transformation [28]. In the present study, we show that NEDD9 function is regulated by the PP2A class of tumour suppressor phosphatases. Identification of NEDD9 as part of this tumour suppressor signalling network underscores the important role that deregulated NEDD9 function plays in cancer progression.

This study has investigated the previously proposed model that PP2A catalyses detachment-induced dephosphorylation of 115 kDa NEDD9 to produce the 105 kDa NEDD9 [7]. However, using siRNA-mediated knock-down of PP2A to allow detection of the specific PP2A mediated effects, we have shown that PP2A does not catalyse detachment-induced dephosphorylation of 115 kDa NEDD9. Indeed, loss of 115 kDa NEDD9 appeared to be more rapid subsequent to PP2A knock-down. Instead, our data suggest that PP2A is likely to regulate phosphorylation modifications of NEDD9 that are discrete from the events required to interconvert 105 kDa and 115 kDa NEDD9. Functionally, we have shown that PP2A suppresses NEDD9-mediated adhesion dynamics that underlie cell spreading and reduces the NEDD9-mediated switch to mesenchymal morphologies. We have further established that NEDD9 and PP2A interact in vivo via the NEDD9 SRR domain. We propose that increased levels of NEDD9—either following NEDD9 induction, or in invasive cancers with increased NEDD9 expression [5]—may exceed the PP2A molecules available for interaction, thus leading to increased cell spreading and mesenchymal morphology. The combination of elevated NEDD9 and decreased PP2A via either siRNA-mediated knock-down or expression of dominant negative PP2A may therefore further shift this balance, resulting in additional increases in NEDD9-mediated spreading. Given that inhibition of PP2A activity alone promotes cell spreading (Fig. 5C) it was possible that PP2A depletion and NEDD9 may stimulate cell spreading via parallel pathways. However, we show that mutation of NEDD9 S369 to alanine abrogates dominant negative PP2A-induced increases in cell spreading. Therefore, this reveals that NEDD9 and PP2A functionally interact to regulate cell spreading. Moreover the finding that the S369D phospho-mimetic construct mimics the effects of PP2A knock-down, suggests that the phosphorylation of this residue is critical to NEDD9 function in adhesion dynamics that lead to cell spreading. Notably, as expression of the S369A mutant protein alone stimulates spreading equivalent to wild-type GFP.NEDD9, there are likely to be other components of the NEDD9 sequence in addition to S369 that regulate cell spreading.

Targeting of PP2A function via dominant negative approaches [19,29] and siRNA-mediated knock-down (present study) leads to increased cell spreading. In addition to the interaction with NEDD9 demonstrated in the present study, PP2A interacts with other adhesion regulators including beta integrins [30,31], the integrin-adhesion associated protein paxillin [20] and the NEDD9-related molecule p130Cas [32]. Each of these proteins contributes to the regulation of adhesion dynamics [33] and collectively these data therefore point to a role for PP2A in regulating the integrin-adhesion dynamics that determine mesenchymal invasion. Moreover PP2A appears to collaborate with NEDD9 to determine the extent of cell spreading and adoption of mesenchymal morphology. A role

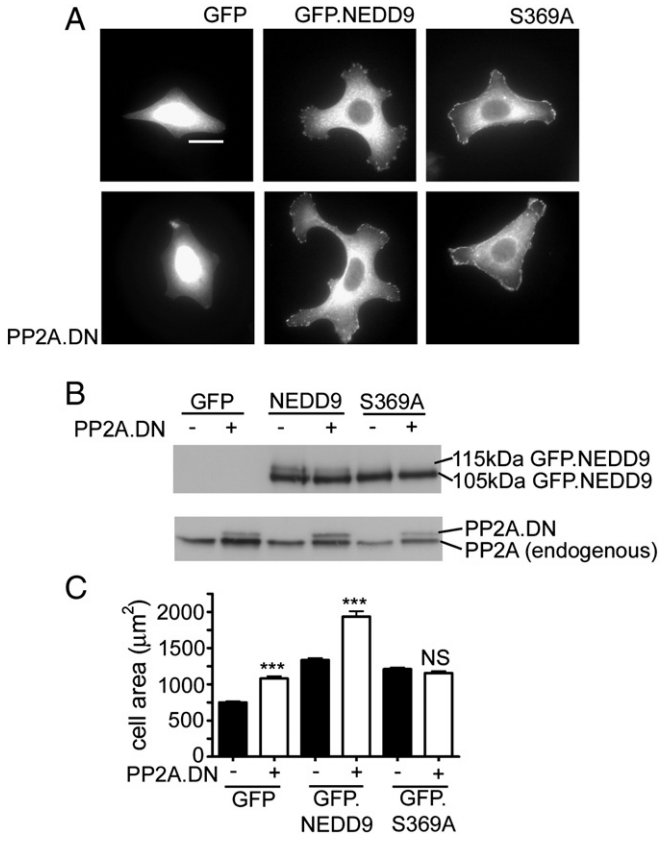

Fig. 5. Mutation of NEDD9 S369 abrogates spreading induced by dominant negative PP2A. A. Images of MCF-7 cells transfected with the indicated expression constructs. Cells in the bottom row were co-transfected with plasmid encoding dominant negative PP2A (PP2A.DN). Scale bar 20 μm. B. Western blot analysis of MCF-7 cells transfected with the indicated expression constructs. Cells were co-transfected with GFP expression constructs and either empty vector control (−) or PP2A.DN (+) as indicated. C. Average area of GFP positive cells. Data are calculated from triplicate repeats on separate occasions (~50 cells for each condition per experiment). Values were compared using one-way ANOVA with Tukey's Multiple Comparison test, ***$p<0.001$, NS = not significant.

for PP2A as a suppressor of cell invasion is particularly interesting, given its role as a tumour suppressor. The switch to an invasive, metastatic phenotype is one of the key features of tumour progression and an increasing number of studies have identified NEDD9 as an important metastasis promoter in a range of tumour types [5]. Moreover, NEDD9-mediated invasion is stimulated following inactivation of another tumour suppressor molecule, LKB1 [34]. Together our data suggest that PP2A may serve as a brake on NEDD9 mediated functions in normal cells and that following cell transformation and PP2A loss, NEDD9 promotion of mesenchymal invasion may be switched on.

The emerging importance of NEDD9 as a regulator of mesenchymal invasion means that this protein is an attractive target for novel anti-cancer therapies. However, as NEDD9 is a scaffold that mediates protein–protein interaction, direct targeting of the NEDD9 protein for therapeutic purpose may be technically challenging. An alternative approach is to target enzymes such as PP2A that regulate NEDD9. The PP2A activator molecule, FTY270/fingolimod, has shown great promise for the treatment of malignancies [35,36]. Activating mutations of the oncogene c-KIT inhibited PP2A and reversal of this with the PP2A activator FTY270 led to the conclusion that PP2A inhibition is essential for c-KIT mediated tumorigenesis [36]. Thus it may be relevant that a recent study established that NEDD9 is also a c-KIT target [37]. In future studies it will be of significant interest to determine whether PP2A activation may also have efficacy against NEDD9-mediated invasion in vivo.

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