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Src Kinase Determines the Dynamic Exchange of the Docking Protein NEDD9 at Focal Adhesions*

Peta Bradbury,[1, 2] Cuc T Bach,[1] Andre Paul,[1] and Geraldine M O'Neill[1, 2]

[1]From the Children's Cancer Research Unit, Kids Research Institute at The Children's Hospital at Westmead, NSW, 2145

[2]Discipline of Paediatrics and Child Health, University of Sydney, NSW, 2006

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To whom correspondence should be addressed: Geraldine M. O'Neill, Children's Cancer Research Unit, The Children's Hospital at Westmead, Locked Bag 4001, Westmead, 2145, Australia Tel.: 61 2 98451206; Fax: 61 2 98453078; Email: geraldine.oneill@health.nsw.gov.au

Key Words: NEDD9; focal adhesion; FRAP; Src kinase; FAK; migration

Background: Dynamic exchange provides a mechanism for rapidly reorganizing macromolecular structures.
Results: Exchange of the focal adhesion targeted protein NEDD9 between the cytoplasm and focal adhesions is faster in the absence of Src kinase activity.
Conclusion: Src kinase modulates the transit time of NEDD9 at focal adhesion sites.
Significance: This is a new function for Src kinase in cell migration.

ABSTRACT

Dynamic exchange of molecules between the cytoplasm and integrin-based focal adhesions provides a rapid response system for modulating cell adhesion. Increased residency time of molecules that regulate adhesion turnover contributes to adhesion stability, ultimately determining migration speed across 2-dimensional surfaces. In the present study we test the role of Src kinase in regulating dynamic exchange of the focal adhesion protein NEDD9/HEF1/Cas-L. Using either chemical inhibition or fibroblasts genetically null for Src together with Fluorescence Recovery After Photobleaching (FRAP), we find that Src significantly reduces NEDD9 exchange at focal adhesions. Analysis of NEDD9 mutant constructs with the two major Src interacting domains disabled revealed the greatest effects were due to the NEDD9 SH2 binding domain. This correlated with a significant change in 2D migratory speed. Given the emerging role of NEDD9 as a regulator of focal adhesion stability, the time of NEDD9 association at the focal adhesions is key in modulating rates of migration and invasion. Our study suggests that Src kinase activity determines NEDD9 exchange at focal adhesions and may similarly modulate other focal adhesion-targeted Src substrates to regulate cell migration.

Cell adhesion to the extra-cellular matrix is an essential component of cell migration and is chiefly determined by integrin receptor binding to extra-cellular matrix ligands. Ligand-bound and cross-linked integrins, together with the associated signalling molecules and polymerized actin filaments in the cell interior, comprise specialized adhesion sites known as focal adhesions. These sites both tether the cells to the underlying matrix and transmit signals bi-directionally between the matrix and the cell cytoplasm (1). During cell motility, focal adhesions must form and turnover, allowing grip and release of the underlying matrix and translocation of the cell bodies. Rapid exchange of proteins between focal adhesions and the cytoplasm (measured in time scales of seconds) facilitates the reorganization of the macromolecular focal adhesions in response to conditions that favour migration (2,3). The time with which focal adhesion stabilizing molecules reside in the focal adhesion (residency time) therefore represents an important mechanism to modulate adhesion to the extra-cellular matrix and consequently migratory behaviour.

The balance of focal adhesion assembly versus turnover determines migration speed across 2-dimensional (2D) planar surfaces (4-7). This is a biphasic relationship, where either too little or too much adhesion slows 2D cell speed, and maximal migration speeds are observed at intermediate

attachment strengths (8,9). Thus conditions which alter the turnover rate of focal adhesions can correspondingly tune cell migration speed. Investigations of a number of focal adhesion molecules have confirmed that many serve to regulate focal adhesion turnover rates (10). While a number of molecules stimulate focal adhesion turnover, our recent data have revealed that the focal adhesion associated molecule NEDD9/HEF1/Cas-L instead stabilizes focal adhesions and can thus tune 2D migration speed (11,12).

NEDD9 is a docking protein at focal adhesions that is subject to extensive phosphorylation modification by Src kinase (13). NEDD9 encompasses multiple protein-protein interaction domains that facilitate the docking of interacting partner proteins, stimulating a variety of cellular processes, including a prominent pro-migratory/pro-metastatic role (13,14). Recruitment to focal adhesions is achieved through interaction with focal adhesion kinase (FAK) resident at the focal adhesions (15). FAK then phosphorylates a tyrosine motif (DYDY) in the NEDD9 c-terminal domain that creates a binding site for Src (the Src binding domain SBD) (13). Once docked, Src phosphorylates up to 13 consensus tyrosine phosphorylation sites in the NEDD9 substrate binding domain (SH2BD). This creates docking sites for SH2-domain containing partner proteins and is an important determinant of NEDD9 regulated cell migration (12,16-18).

In addition to the well-established role for Src phosphorylation in stimulating NEDD9-mediated signalling cascades, we hypothesized that NEDD9 phosphorylation by Src may also regulate the residency time at focal adhesions. Increased opportunities for interactions between NEDD9 and other focal adhesion molecules as a result of extensive phosphorylation by Src could increase the time in which NEDD9 is tethered at the focal adhesions by stabilizing interactions with partner molecules. While there have been a handful of studies investigating the exchange rates of focal adhesion molecules, at present there is little understanding of how this is controlled. We have employed Src/Yes/Fyn (Src)-/- mouse embryo fibroblasts (MEFs) and NEDD9 -/- MEFs to investigate the role of Src kinase in NEDD9 dynamic exchange at focal adhesions. Our data indicate that Src regulates the transit time of NEDD9 at focal adhesions.

EXPERIMENTAL PROCEDURES

Cells and cell culture - Src/Yes/Fyn (SYF-/-) null and FAK -/- mouse embryo fibroblasts (MEFs) were from American Type Culture Collection (ATCC) and NEDD9 (NEDD9-/-) MEFs have been described (11). All lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with either 10% foetal bovine serum (FBS) (Life Technologies), 1% penicillin/streptomycin (Invitrogen) and 2μg/ml fungizone (Life Technologies) or 15% FBS and 1% antibiotic/antimycotic (Life Technologies), at 37^0C and 5% CO_2.

Antibodies - Monoclonal anti-NEDD9, polyclonal anti-FAK (Cell Signalling Technology); monoclonal anti-paxillin and p130Cas (BD Transduction Laboratories); anti-Src (Clone GD11) (Millipore); monoclonal anti-GFP (Roche), monoclonal anti-GFP, polyclonal anti-Paxillin pY118 (Life Technologies) anti-β-actin, anti-HSP-70 (Sigma); horseradish peroxidase-conjugated goat secondary antibodies for immunoblot analysis (Amersham Pharmacia Biotech) and; Cy3-conjugated donkey anti-rabbit and Cy5-conjugated donkey anti-mouse IgG secondary antibodies for immunofluorescence (Jackson Immunoresearch).

Plasmids and protein expression - NEDD9 fused to GFP has been previously described (19). QuikChange kit (Stratagene) was used to mutate NEDD9 Y629 and Y631 to phenylalanines (NEDD9.DFDF). Primer pairs: forward 5' GCTGGATGGATGACTT3', reverse 5'TCGAAGTCATCCATCCAGC 3' and forward 5'CGATTTCGTCCACCTACAGGG3' and reverse 5' CCCTGTAGGTGGACGAAA3'. SH2BD domain deletion was achieved by synthesizing the sequence encoding the first 67 amino acids (M1-M67), flanked by EcoR1 and AvaI restriction sites, then joined by restriction enzyme-mediated ligation with the NEDD9 beginning at S358 (deleting the intervening 290 residues). The mutant sequence was sub-cloned into the same GFP vector backbone as the wild-type sequence. All constructs were confirmed by DNA sequencing. Transient MEF cell transfection was achieved using a nucleofector (Amaxa) and MEF1 nucleofector kit (Lonza).

Immunoprecipitation, immunoblot and immunofluorescence - Immunoprecipitation was performed using 1 mg of protein lysate extracted in NP-40 lysis buffer (50 mM Tris (pH 7.5) 150 mM NaCl, 5 mM EDTA, 1% NP-40) freshly supplemented with protease inhibitors (1 mM

NA_3VO_4, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin). Protein G agarose was swollen in 10 mM HEPES (pH 7.4) on ice for 30 min, then washed in PBS and resuspended at a concentration of 4 mg/ml in NP- lysis buffer supplemented with protease inhibitors. Cell lysates were pre-cleared with 4 mg of agarose and mixed end-over-end at 4°C for 1 hour. Pre-cleared lysates were incubated with anti-GFP (Roche) or mouse IgG for 2 hours at 4°C to which 4 mg of protein G agarose was added and incubated for a further 16 hours. Immunoprecipitates were washed three times with NP-40 lysis buffer (supplemented with inhibitors) and eluted in SDS-PAGE sample buffer. For all other immunobloting PTY lysis buffer protein extraction and immunoblotting were performed as previously described (20). For immunofluorescence cells cultured overnight on glass coverslips were fixed in 4% paraformaldehyde (PFA) and permeabilized in PBS/0.5% bovine serum albumin and 0.2% Triton-X. Coverslips were mounted with Fluorsave (Merck-Millipore). Confocal microscopy was performed using a Leica SP5 confocal, 63 X oil objective. Images were captured using the Leica LAS-AF software. Cells were imaged at a single optical plane corresponding to focal adhesion positions on the ventral surface.

Fluorescence Recovery After Photobleaching (FRAP) - Transfected cells were cultured overnight at 37°C with 5% CO_2 in 35mm glass bottom dishes (MatTek, Boston, MA, USA). Media was then exchanged for CO_2-independent media supplemented with 15% FBS and antibiotic/antimycotic and cultures equilibrated at 37°C for 1 hour. FRAP analysis was performed using either Leica SP5 confocal and 63 X oil objective (NEDD9 mutant constructs) or Olympus FV1000 confocal and 60 X oil objective (SYF-/- and PP2 experiments), both equipped with a 37°C environmental chamber. Focal adhesions were photobleached for 3 seconds with an argon laser at 50%. Images of the bleached area were then recorded every 1.4 seconds over a minimum 60 second period. Recovery rates were calculated and normalised to single control measurements taken per focal adhesion, using either the Leica supported software LAS-AF or the Olympus Fluoview software. FRAP analysis was performed on cells from 3 independent experiments on separate days and then pooled to obtain sufficient numbers of focal adhesions for analysis. Src inhibition with PP2 (Sigma Aldrich) was achieved by incubation with 2μM PP2 overnight prior to analysis. Statistical analysis was performed using GraphPad Prism.

Live cell imaging, migration and spreading analysis - Time-lapse images of transfected cells at low confluence in a 35mm plastic dish were captured using an ORCA ERG cooled CCD camera (Hamamatsu SDR Clinical Technology NSW, Australia) and an Olympus IX81 inverted microscope equipped with a 37°C environmental chamber. Transmitted light images of transfected cells (identified by initial single fluorescence image) were captured every 5 minutes over 6 hours. Cells undergoing division or apoptosis were excluded from analysis. Nuclear translocation was tracked in the time-lapse image stacks using Metamorph V6.3 software (Molecular Devices).

RESULTS

Src regulates NEDD9 exchange at focal adhesions. We first confirmed that NEDD9 localization to focal adhesions does not require Src kinase activity by transfecting Src-/- MEFs with GFP-tagged NEDD9. NEDD9 robustly localized to focal adhesions in a similar pattern to that observed in NEDD9-/- MEFs, with strong concordance between the focal adhesion marker paxillin and GFP.NEDD9 sub-cellular distribution (Fig. 1A). NEDD9 is detected as two phospho-forms by western blot (21,22) and exogenous GFP.NEDD9 similarly produces a doublet in NEDD9-/- and Src-/- MEFs (Fig. 1A). The small decrease in the upper form of NEDD9 in the Src-/- MEFS was inconsistent between experiments (data not shown).

We next compared GFP-tagged NEDD9 exchange at focal adhesions between Src-/- and NEDD9-/- MEFs by Fluorescence Recovery After Photobleaching (FRAP). For uniformity, all analyses were focused on focal adhesions in regions of protruding cell membrane. Notably, the recovery of GFP.NEDD9 appeared faster in the Src -/- MEFs (Figs 1B and 1C). Quantification of recovery rates confirmed that they were indeed significantly faster (Fig. 1D). To discount the possibility that the differences seen might be due to different cell backgrounds (Src-/- versus NEDD9-/- MEFs) GFP.NEDD9 recovery was next measured in NEDD9-/- MEFs treated with PP2, a chemical inhibitor of Src kinase. Importantly, NEDD9 fluorescence recovery in the presence of PP2 was significantly increased when compared to untreated NEDD9 -/- MEFs (Figs 1C and 1D). Since the Src-/- MEFs express endogenous

NEDD9, to rule out that the difference reflect changes in the total amount of NEDD9 protein, we next compared the dynamics of GFP.NEDD9 in NEDD-/- MEFs versus matched wild-type MEFs. This revealed identical recovery kinetics between the NEDD9-/-MEFs and the wild-type MEFs (Figure 1E). Together these data suggest an important role for Src in determining NEDD9 exchange at focal adhesions.

For comparison, we examined GFP.NEDD9 with the C-terminal domain deleted (NEDD9.ΔCT). This domain encompasses the focal adhesion targeting domain (23) and is required for focal adhesion localization (24). Cells were co-transfected with mcherry-tagged paxillin to identify the focal adhesion region of interest for photobleaching (Figs 2A and 2B). As anticipated, the NEDD9.ΔCT construct exhibited faster exchange in the region of the focal adhesions (Figs 2C and 2D). However, this did not simply reflect free diffusion, since the same analysis of unfused GFP at focal adhesions marked by mcherry paxillin showed an ~ 10-fold higher exchange rate (GFPNEDD9.ΔCT: $K = 0.35 \pm 0.018$ versus GFP: $K = 2.98 \pm 0.24$, $^{***}p<0.001$, Students' *t*-test). Consequently, despite the lack of the focal adhesion targeting domain (thereby abrogating GFP-positive adhesions), the molecular dynamics were slowed around the focal adhesion. Together this data suggests that localization (concentrated appearance at a focal adhesion) is separable from the rate of exchange between the focal adhesion juxtamembrane region and the cytoplasm.

The NEDD9 SH2 binding domain dominantly regulates exchange. Src stimulates focal adhesion turnover (10), and regulates actin filament dynamics (25) in turn controlling acto-myosin force that determines adhesion molecule exchange (3). Potentially, therefore, faster NEDD9 exchange in the absence of Src activity may have been secondary to altered actin filament dynamics or Src-dependent focal adhesion turnover. To rule this out, we quantified the role of the two direct NEDD9 Src target sites: the SBD (DYDY) and the SH2BD (26). First, the SBD tyrosines were mutated to phenylalanine, to prevent phosphorylation (DFDF) (Fig. 3A). Note that NEDD9 does not contain the additional Src SH_3 binding that forms the Src bipartite binding domain in other Cas family proteins (13) and so only binds Src via the tyrosine motif. NEDD9.DFDF generates the same doublet pattern on western blot as seen for the wild-type sequence (Fig. 3A). Immunofluorescence confirmed co-localization of the mutant protein with paxillin-positive focal adhesions at the cell periphery and ventral surface (Fig. 3B). FRAP analysis of GFP.NEDD9.DFDF positive focal adhesions revealed a significantly increased fluorescence recovery rate compared to wild type NEDD9 (Figs 4A-4C). Thus phosphorylation of the DYDY motif and subsequent Src kinase docking may slow the rate of NEDD9 molecular exchange at focal adhesions.

Next, we analysed a NEDD9 SH2BD deleted mutant (NEDD9ΔSH2BD). As this also deletes the NEDD9 antibody-binding epitope, anti-p130Cas antibodies which bind a conserved epitope (19) were used to confirm expression of the truncated protein (Fig. 3A). Importantly, loss of the substrate binding domain does not affect NEDD9 targeting to the focal adhesions (Fig. 3B). GFP-tagged NEDD9ΔSH2BD co-localized at all regions of paxillin-positive focal adhesions (Fig. 3B). FRAP analysis of this construct revealed a striking increase in the rate of fluorescence recovery (Figs 4A-4C). Notably, this was also significantly faster than the recovery rate of NEDD9.DFDF (Fig. 4C). We then questioned whether the NEDD9 mutant proteins exhibited an altered mobile fraction, defined as the maximum plateau of fluorescence recovery. While analysis revealed a significant difference between the maximum plateaus of the NEDD9ΔSH2BD versus NEDD9.DFDF and NEDD9 (Fig. 4D), the magnitude of the difference was very small (80% and 82% versus 80% and 79%). Thus in each case ~ 20% of the total pool remains immobile, agreeing with earlier descriptions of the stable focal adhesion fraction over a similar time course (27). Notably, the difference between GFP.NEDD9 in the NEDD9-/- MEFs versus the Src-/- MEFs was considerably larger (Fig. 1C). The relatively smaller difference between the mutant forms and the PP2-treated cells with wild-type NEDD9 suggests differences observed in the Src-/- MEFs may be cell type specific and suggest that the most important effects of Src may be on exchange. Our data suggest a hierarchy of Src kinase effects: the substrate binding domain has the greatest effects on NEDD9 dynamics at focal adhesions, while the Src SH2 binding motif has a more limited role.

Interaction with FAK regulates NEDD9 focal adhesion targeting. Given the differences in molecular exchange between wild-type NEDD9, NEDD9ΔCT, NEDD9.DFDF and NEDD9ΔSH2BD we questioned whether these

differences might reflect differential interaction with NEDD9 partner molecules. As FAK is a well-established partner of NEDD9 (13), FAK interaction with each of the NEDD9 constructs was assessed by co-immunoprecipitation. This revealed that NEDD9, NEDD9.DFDF and NEDD9ΔSH2BD show equivalent and robust interaction with FAK (Figure 5A). By contrast considerably less FAK co-immunoprecipitates with NEDD9ΔCT. Thus we next confirmed that FAK is required for NEDD9 targeting to focal adhesions, by analysing GFP.NEDD9 sub-cellular distribution in FAK-/- fibroblasts. This revealed that GFP.NEDD9 does not target to focal adhesions in the absence of FAK expression (Figure 5B). Importantly, exogenous expression of FAK restores NEDD9 targeting to focal adhesions (Figure 5B). Based on these findings we suggest that the interaction between NEDD9ΔCT and FAK may attenuate NEDD9ΔCT dynamics but requires the NEDD9 c-terminal domain in order to anchor NEDD9 at the focal adhesions.

NEDD9 SH2BD affects focal adhesion signalling. Next we questioned the effect of the altered rates of molecular exchange on focal adhesion morphology and signalling. Comparison of the total number of focal adhesions revealed that neither NEDD9.DFDF nor NEDD9ΔSH2BD altered the number of focal adhesions per cell (Figure 6A and 6B). However, focal adhesions in cells expressing NEDD9ΔSH2BD were significantly larger (Figure 6C). To investigate whether expression of the NEDD9 mutant constructs altered focal adhesion signalling, we analysed paxillin phosphorylation at focal adhesions. Ratio imaging of focal adhesions co-immunostained with antibodies to total paxillin and paxillin phosphorylated at Y118 suggested that paxillin phosphorylation at focal adhesions is reduced in cells expressing NEDD9.DFDF and NEDD9ΔSH2BD (Figure 6A). Semi-quantitative measurement of the ratio of phosphorylated paxillin at focal adhesions confirmed this observation (Figure 6D).

The SH2BD mediates cell migration. Faster focal adhesion turnover due to the absence of NEDD9 generates faster 2D cell migration speeds in NEDD9 -/- MEFs; conversely, in the presence of NEDD9 focal adhesions are stabilized resulting in slower migration speeds (11). Agreeing with this earlier data, exogenous NEDD9 expression in the NEDD9-/- MEFs slows cell progression (Fig. 7A), and individual cell migration speed (Fig. 7B). We then hypothesized that conditions of more rapid NEDD9 exchange at focal adhesions should equate to increased cell migration speed. Indeed, cells expressing NEDD9ΔSH2BD displayed a greater mean squared displacement (MSD) than either GFP.NEDD9 or GFP.NEDD9DFDF (Fig. 7C) and significantly faster cell speeds (Fig. 7D). These findings agree with other studies that have highlighted the role of the NEDD9 SH2BD in regulating cell migration (12,16-18).

DISCUSSION

NEDD9 localization to focal adhesions is key to the function of this protein in cell migration however the molecular regulation of NEDD9 exchange at focal adhesions has not been previously explored. In the present study, we found that Src kinase activity is required to slow NEDD9 exchange at focal adhesions and deletion of the NEDD9 domain that is targeted by Src significantly increases the speed of NEDD9 exchange. We therefore propose that Src phosphorylation of NEDD9 is an important determinant of NEDD9 residency time at focal adhesions.

While it has long been known that focal adhesions are regulated via assembly and turnover, the constant molecular exchange between focal adhesion-associated molecules and their cytoplasmic counterparts is a more recently recognized control mechanism (2). There is precedence for signalling-dependent regulation of focal adhesion residency time. Calcium flux slows FAK recovery, thus prolonging FAK association at focal adhesions (28), while phosphorylation of the FAK auto-phosphorylation site (Y397) was sufficient to increase residency time (29). We have now shown that Src regulates the residency time of NEDD9 at focal adhesions. Since Src is a major regulator of focal adhesion disassembly, including directly regulating FAK activity and acto-myosin contractility, it was necessary to test whether the effects on NEDD9 were either direct or secondary to other Src targets. Importantly, the demonstration that the NEDD9 SH2BD is required to regulate the rate of NEDD9 molecular exchange confirmed that the effects seen were direct.

Focal adhesion components examined to date exist in at least two states, an immobile fraction and a fraction that exchanges between the cytoplasm and the focal adhesion. Recent reports have also suggested a third behaviour, where attenuated diffusion of focal adhesion components in the juxtamembrane region close to the focal adhesions serves as a pool of molecules for

exchange with the focal adhesions (27). Proteins that freely diffuse at focal adhesions should have a mobile fraction of 100% and a reduced mobile fraction suggests strong binding to fixed components (30). We found that NEDD9 has a relatively high mobile fraction, with ~ 80% of the total pool recovered post-bleaching. The related Cas family protein p130Cas (26) has also been shown to have a high mobile fraction, as compared for example to paxillin (31). Interestingly, paxillin contains multiple LIM domains that mediate direct binding to actin (32). Conceivably, the reduced mobility of paxillin at focal adhesions may be due to paxillin binding to a stable actin core. By contrast, NEDD9 and p130Cas may not interact with stable structural elements of the focal adhesions and hence have relatively high mobile fractions. Notably, the magnitude of difference between the mobile fractions of the NEDD9 mutant proteins was very small, suggesting that the NEDD9 SBD and SH2BD have limited impact on the total fraction of mobile NEDD9 at focal adhesions.

The rate of protein recovery at the focal adhesions depends on the stability of the interaction with proteins in the focal adhesions (30). In line with this, deletion of the NEDD9 c-terminal domain reduces interaction with FAK and this mutant exhibits rapid exchange in the vicinity of the focal adhesions. We note that while mutation of the SBD resulted in significantly faster recovery rates than observed for the wild-type molecule, deletion of the SH2BD resulted in an even faster recovery rate than the SBD mutant. Moreover, the SBD mutation had no significant effect on NEDD9-mediated 2D cell migration, but the SH2BD mutation significantly altered the NEDD9-mediated migration effects. Thus, our data suggest that Src-mediated phosphorylation of sites in the NEDD9 SH2BD may stabilize interactions between NEDD9 and proteins resident at the focal adhesions. We have previously shown that NEDD9 stabilizes focal adhesions (11) and therefore we suggest that increased residency time of NEDD9 at the focal adhesions facilitates NEDD9-mediated focal adhesion stabilization. Similarly, increased FAK residency time – mediated by Y397 phosphorylation (29) – promotes the focal adhesion disassembly function of FAK by allowing increased time for FAK's action on its cognate partners. Thus together, our data suggest that the SH2BD serves an important function in regulating the rate of NEDD9 exchange at the focal adhesions, thereby determining focal adhesion stability and subsequent cell migration speeds.

Given that there are multiple consensus tyrosine phosphorylation sites contained within the NEDD9 substrate domain, it is possible that either multiple tyrosine phosphorylations are required for stabilizing NEDD9 interaction at the focal adhesions or individual phosphorylations may be important. A high stringency scan for putative SH2 interactors at the Scansite website reveals predicted motifs for a number of proteins including Abl, Itk and Lck kinases and the adaptor molecules Crk, Nck and Grb2 (33). Our data suggests that interaction with one or more of these proteins, and potentially other as yet unidentified partners, following Src-mediated phosphorylation determines the stability of NEDD9 association at focal adhesions.

The present study expands the previously ascribed role for Src kinase in focal adhesion turnover (25), to a novel role in regulating molecule exchange rates at focal adhesions. Given the large array of Src substrates at focal adhesions (34), we suggest that this may prove to be a more general function of Src at focal adhesions, identifying a new biological role for this important enzyme (7,12,16-18).

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FOOTNOTES

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[1] To whom correspondence should be addressed: Children's Cancer Research Unit, The Children's Hospital at Westmead, Locked Bag 4001, Westmead, 2145, Australia Tel.: 61 2 98451206; Fax: 61 2 98453078; Email: geraldine.oneill@health.nsw.gov.au

[2]Discipline of Paediatrics and Child Health, University of Sydney, NSW, 2006

[3]The abbreviations used are: NEDD9, Neural pre-cursor cell expression developmentally down-regulated 9; FRAP, fluorescence recovery after photo-bleaching; FAK, focal adhesion kinase; SH2, Src-homology 2 domain.

FIGURE LEGENDS

FIGURE 1. Src regulates NEDD9 molecular exchange. (a) Confocal images of GFP.NEDD9 in NEDD9 -/- and Src -/- MEFs. Paxillin immunostaining identifies focal adhesions (centre panels) and NEDD9/paxillin co-localisation is shown in merged images (right panels). Scale bars = 20 μm. Immunoblots were probed with indicated antibodies. Antibodies to HSP-70 used as loading control. (b) Representative focal adhesions prior to photobleaching (left panel), immediately post-photobleaching (0") and throughout fluorescence recovery (5-40"). (c) Recovery kinetics of the indicated GFP-tagged constructs. Data plots compare normalised fluorescence recovery for each construct. (d) Rates of GFP.NEDD9 fluorescence recovery post photobleaching for the indicated conditions. Data represent the mean values of >13 focal adhesions per construct and error bars show SEM. ** $p<0.01$ and *** $p<0.001$, one-way ANOVA with Tukey's Multiple Comparison post-test. (e) Fluorescence recovery of GFP.NEDD9 is identical between NEDD9-/- MEFs (black squares) versus wild-type (WT) MEFs (white squares).

FIGURE 2. FRAP analysis of NEDD9ΔCT. (a) Schematic of NEDD9 wild-type (WT) sequence and C-terminal domain deletion mutant (NEDD9ΔCT). (b) As NEDD9ΔCT does not localize to focal adhesions, cells were co-transfected with mcherry-tagged paxillin to detect focal adhesions. This was used to mask the relevant areas in the GFP images to analyse NEDD9ΔCT dynamics. Shown is an example of mcherry paxillin used to identify relevant areas for photo-bleaching the GFP signal. Scale bars = 5 μm. (c) Fluorescence recovery of GFP.NEDD9 (black squares) versus GFP.NEDD9ΔCT (white squares) in NEDD9-/- MEFs. (d) Fluorescence recovery rates for the indicated constructs. Data represent the mean of >43 focal adhesions per construct and error bars show SEM. *** $p<0.001$, Students' *t*-test.

FIGURE 3. NEDD9 Src SH2 binding motif and substrate binding domain are not required for focal adhesion localization. (a) Schematic of the Src binding site mutant (NEDD9.DFDF) and SH2 binding domain deleted mutant (NEDD9.ΔSH2BD). Cartoons show the SH_3 domain, the substrate binding domain (SH2BD), the serine rich region (SRR) and the C-terminus (CT). The NEDD9 antibody binding epitope (*) and p130Cas antibody cross-reacting epitope (**) are indicated. As the SH2BD encompasses the anti-NEDD9 antibody epitope, anti-p130Cas antibodies (conserved antibody epitope in the C-terminus of NEDD9) were used to confirm expression of NEDD9ΔSH2BD (immunoblots on the right). (b) NEDD9-/- MEFs transfected with the indicated constructs were immunostained with paxillin to identify focal adhesions (centre panels), NEDD9 and paxillin co-localization shown in the merged images (right panels). Scale bars = 20μm.

FIGURE 4. NEDD9 Src SH2 binding motif and substrate binding domain mediate molecular exchange. (a) Representative focal adhesions from NEDD9-/- MEFs expressing the indicated GFP fusion proteins,

shown prior to photobleaching (left panel), following photobleaching (0") and throughout the recovery period (5-60"). Scale bars = 5 μm. (b) The recovery kinetics of the indicated GFP-tagged constructs. Data plots show normalised fluorescence recovery for each construct. (c) Fluorescence recovery rates post-photobleaching for each construct. (d). Average mobile fractions. Note that error bars (SEM) are present, but not visible due to small size. Data represent the mean values of >45 focal adhesions per construct and error bars show SEM; *** $p < 0.001$, *$p < 0.05$, NS = not significant; one-way ANOVA with Tukey's Multiple Comparison test.

Figure 5. NEDD9 interaction with FAK and targeting to focal adhesions. (a) NEDD9-/- MEFs were transfected with the indicated GFP-tagged NEDD9 constructs. Exogenous GFP-tagged proteins were immunoprecipitated (IP) with anti-GFP antibodies and western blots of immunoprecipitates probed with the indicated antibodies (anti-FAK and anti-GFP). Western blots of whole cell lysates (WCL) were probed as indicated to demonstrate protein expression prior to IP. (b) Micrographs show confocal images of FAK-/- MEFs expressing GFP.NEDD9 alone, or cotransfected with an exogenous FAK expression construct (lower panels). Cells were immunostained with paxillin antibodies (centre panels). Scale bars = 20 μm. Immunoblots confirm expression of the exogenous molecules in FAK-/- cells expressing GFP.NEDD9 alone, or in cells reconstituted with FAK. Antibodies to HSP-70 used as loading control.

Figure 6. Focal adhesion phenotypes and signalling. (a) NEDD9-/- MEFs transfected with the indicated GFP-tagged constructs, fixed and co-immunostained for paxillin (red) and phospho-paxillin (pY118, blue). The third column shows the merge of GFP/paxillin/phospho-paxillin staining. Final panels show paxillin phosphorylation represented by ratio imaging. Insets show magnified regions of focal adhesions. Yellow hues indicate areas of increased paxillin phosphorylation. (b) The average number of focal adhesions per cell. (c) The sum of the focal adhesion area expressed as a percentage of the total cell area. Data points represent the average value for each cell. (d) Distribution of the ratios of phosphorylated paxillin at focal adhesions. Data points show the average value for all focal adhesions in each cell and horizontal bars indicate the mean of each population. n>45 cells examined for each parameter. *** $p < 0.001$, NS = not significant; one-way ANOVA with Tukey's Multiple Comparison test.

FIGURE 7. SH2BD mediates regulation of cell migration. (a) Transfected NEDD9-/- MEFs were analysed by time-lapse imaging over a 6 hour period. Mean Squared Displacements (MSD) were calculated from cell trajectories. (b) Plots of individual migration speeds. Horizontal bars indicate the average speed (n>150 cells per construct). *** p<0.0001 Students' *t*-test. (c) MSD calculated for NEDD9-/- MEFs expressing the indicated GFP-fused NEDD9 proteins. (d) Plots of individual migration speeds (n>150 cells per construct). ** p<0.001 one-way ANOVA with Tukey's Multiple Comparison post-test.

FIGURE 1

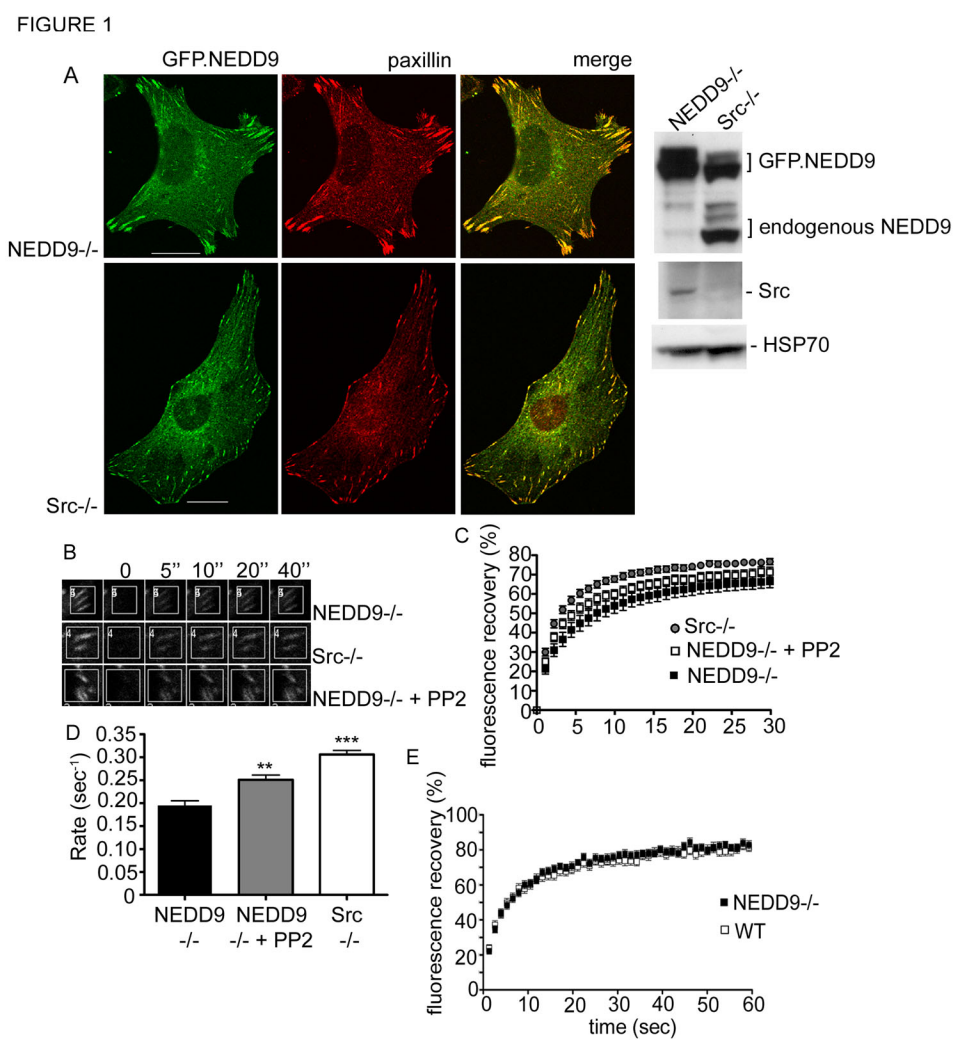

A
GFP.NEDD9
paxillin
merge
NEDD9-/-
Src-/-
NEDD9-/-
Src-/-
] GFP.NEDD9
] endogenous NEDD9
- Src
- HSP70
B
0
5''
10''
20''
40''
NEDD9-/-
Src-/-
NEDD9-/- + PP2
C
fluorescence recovery (%)
Src-/-
NEDD9-/- + PP2
NEDD9-/-
D
Rate (sec-1)
**
***
NEDD9 -/-
NEDD9 -/- + PP2
Src -/-
E
fluorescence recovery (%)
NEDD9-/-
WT
time (sec)

FIGURE 2

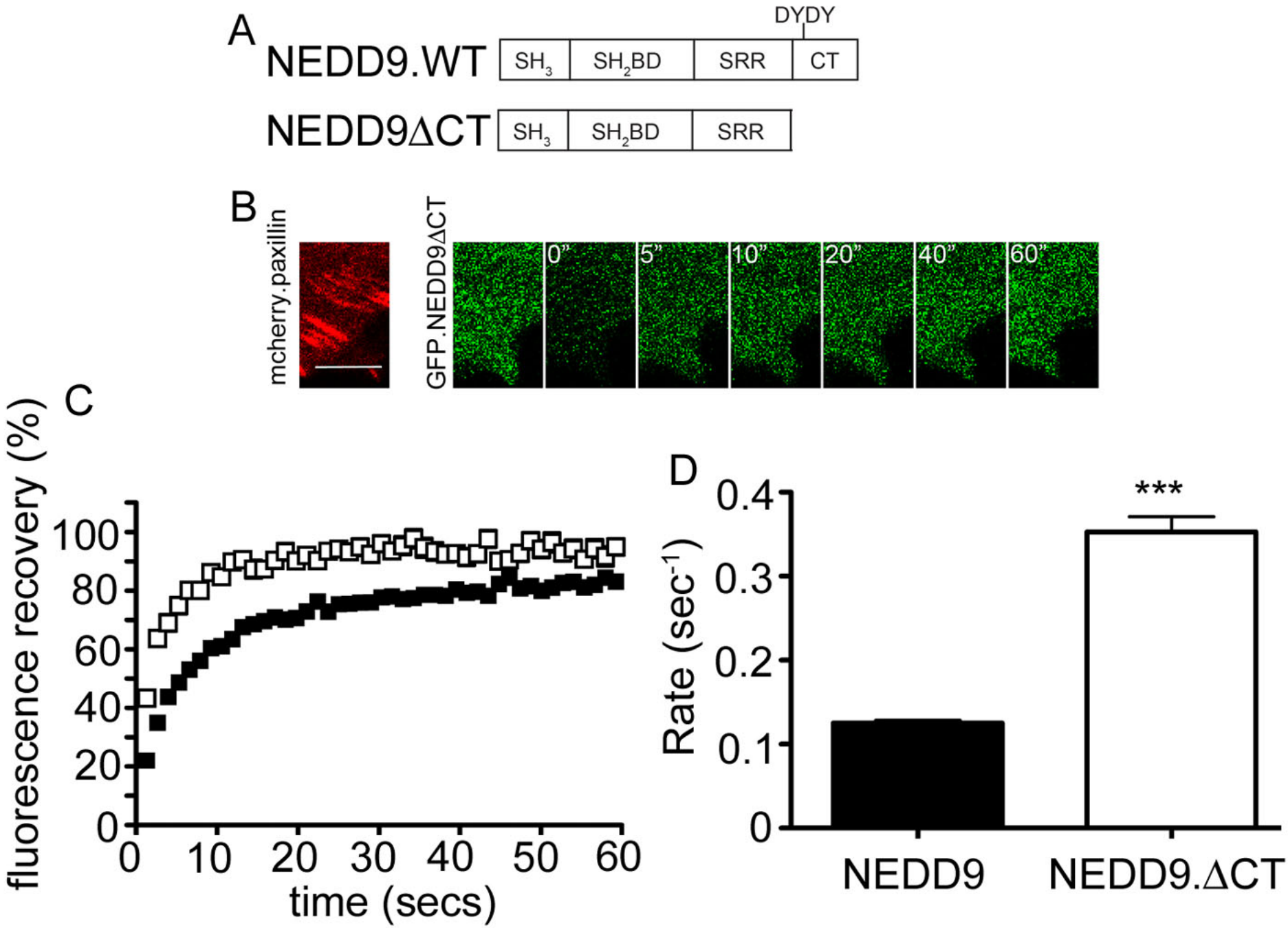

A
DYDY
NEDD9.WT
SH3
SH2BD
SRR
CT
NEDD9ΔCT
SH3
SH2BD
SRR
B
mcherry.paxillin
GFP.NEDD9ΔCT
0"
5"
10"
20"
40"
60"
C
fluorescence recovery (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
time (secs)
D
Rate (sec-1)
0.4
0.3
0.2
0.1
0
***
NEDD9
NEDD9.ΔCT

FIGURE 3

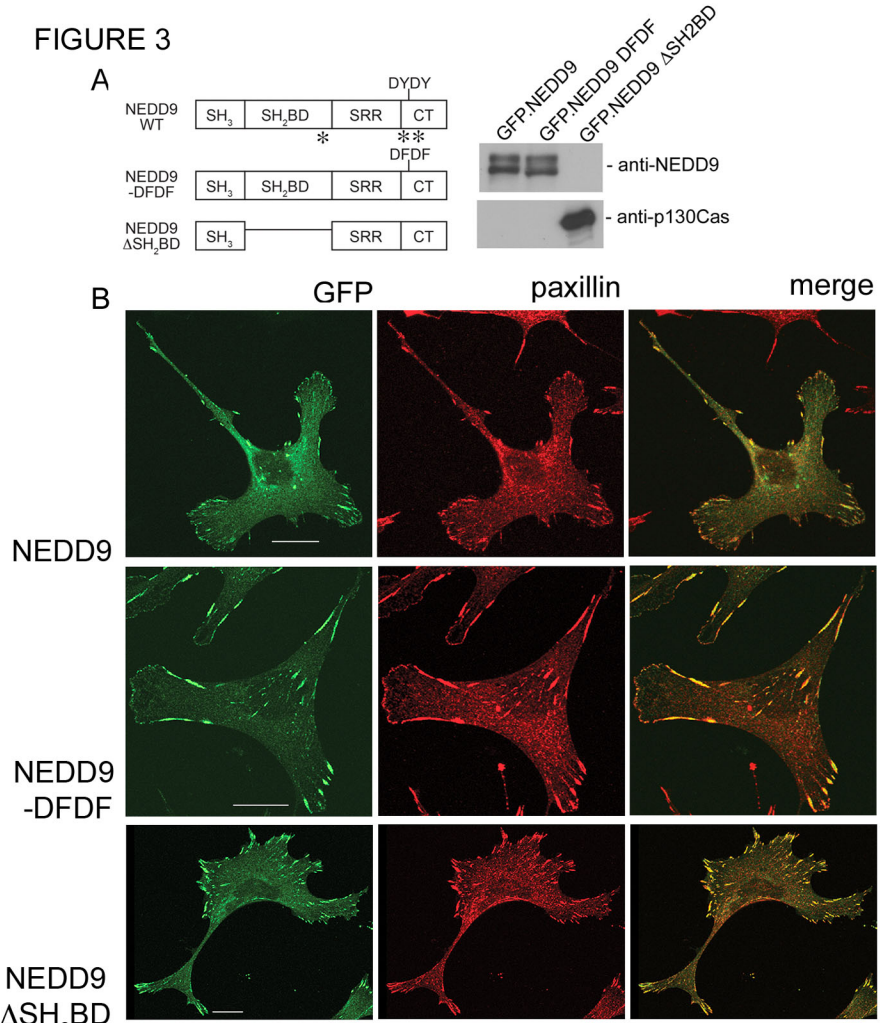

A
DYDY
NEDD9 WT
SH3
SH2BD
SRR
CT
DFDF
NEDD9 -DFDF
NEDD9 ΔSH2BD
GFP.NEDD9
GFP.NEDD9 DFDF
GFP.NEDD9 ΔSH2BD
- anti-NEDD9
- anti-p130Cas
B
GFP
paxillin
merge
NEDD9
NEDD9 -DFDF
NEDD9 ΔSH2BD

FIGURE 4

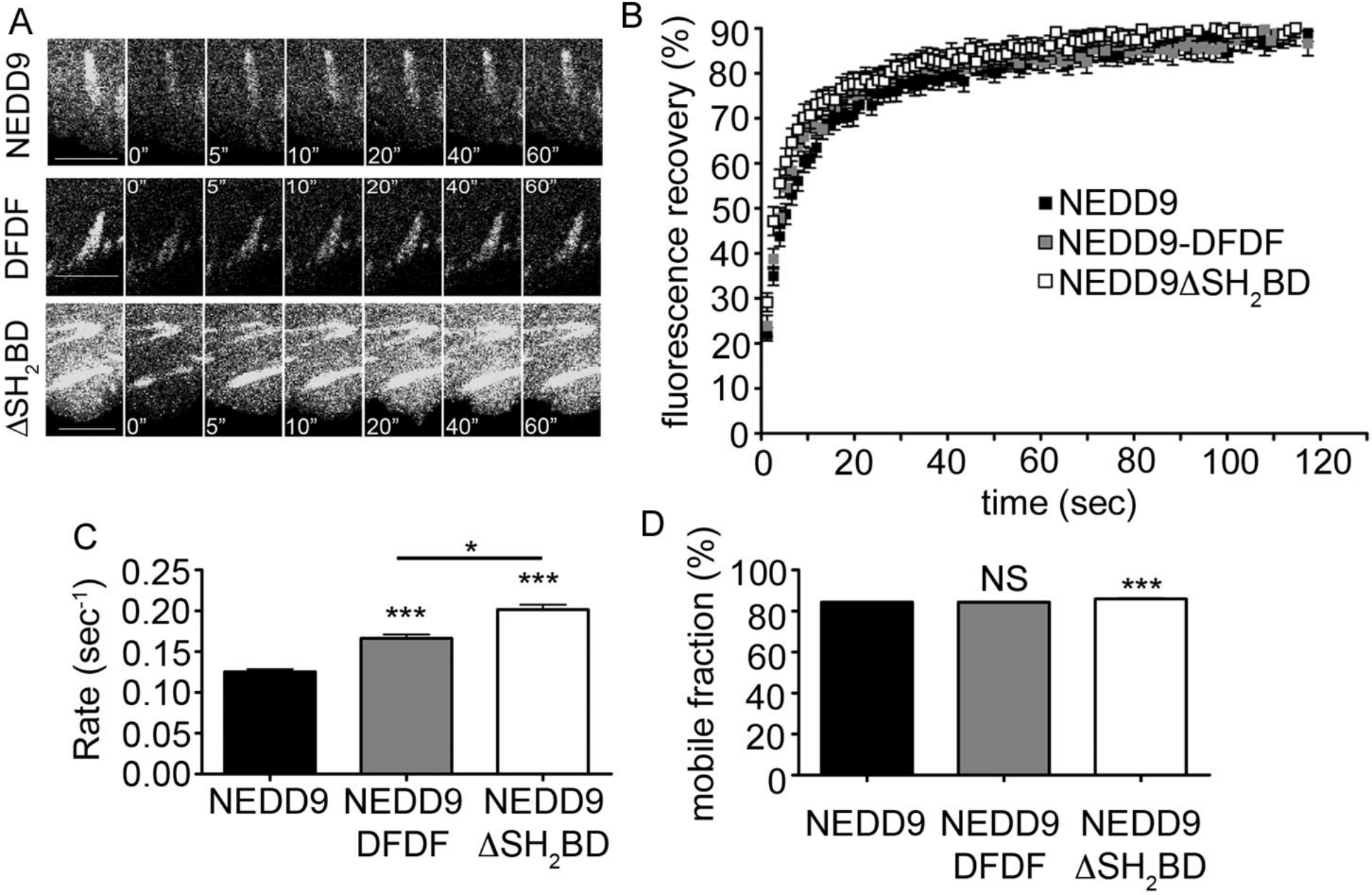
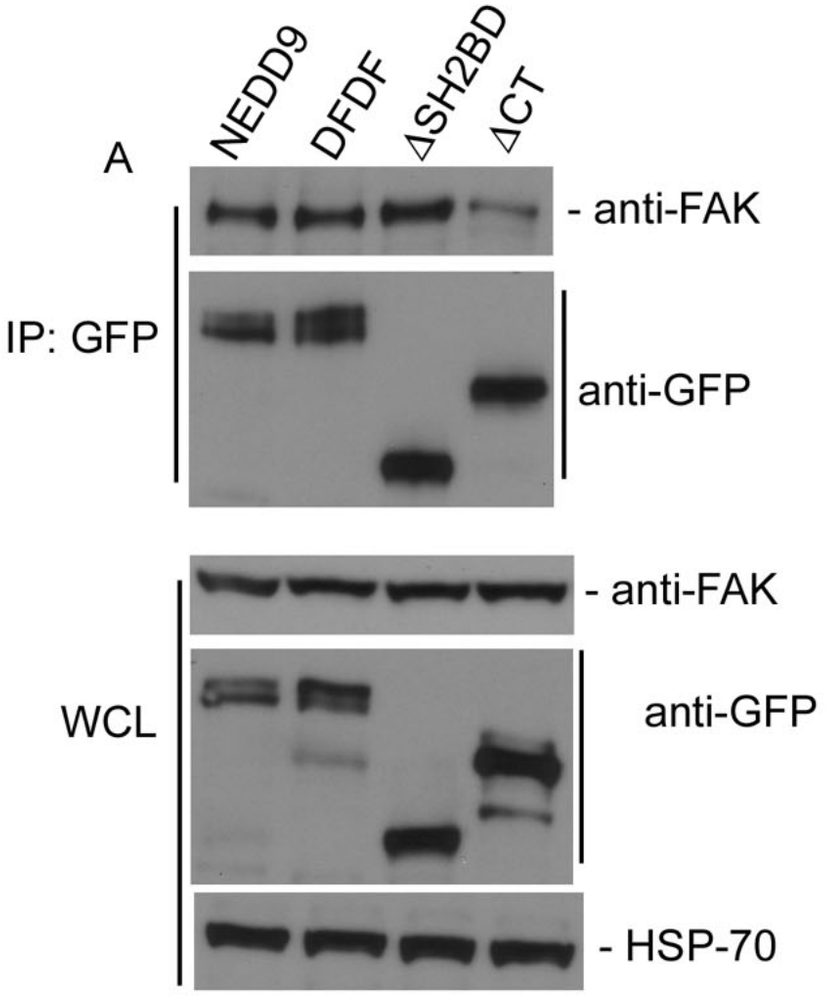

A
NEDD9
DFDF
ΔSH2BD
0"
5"
10"
20"
40"
60"
B
fluorescence recovery (%)
time (sec)
NEDD9
NEDD9-DFDF
NEDD9ΔSH2BD
C
Rate (sec-1)
*
***
***
NEDD9
NEDD9 DFDF
NEDD9 ΔSH2BD
D
mobile fraction (%)
NS
***
NEDD9
NEDD9 DFDF
NEDD9 ΔSH2BD

FIGURE 5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NEDD9
DFDF
ΔSH2BD
ΔCT
- anti-FAK
IP: GFP
anti-GFP
- anti-FAK
WCL
anti-GFP
- HSP-70

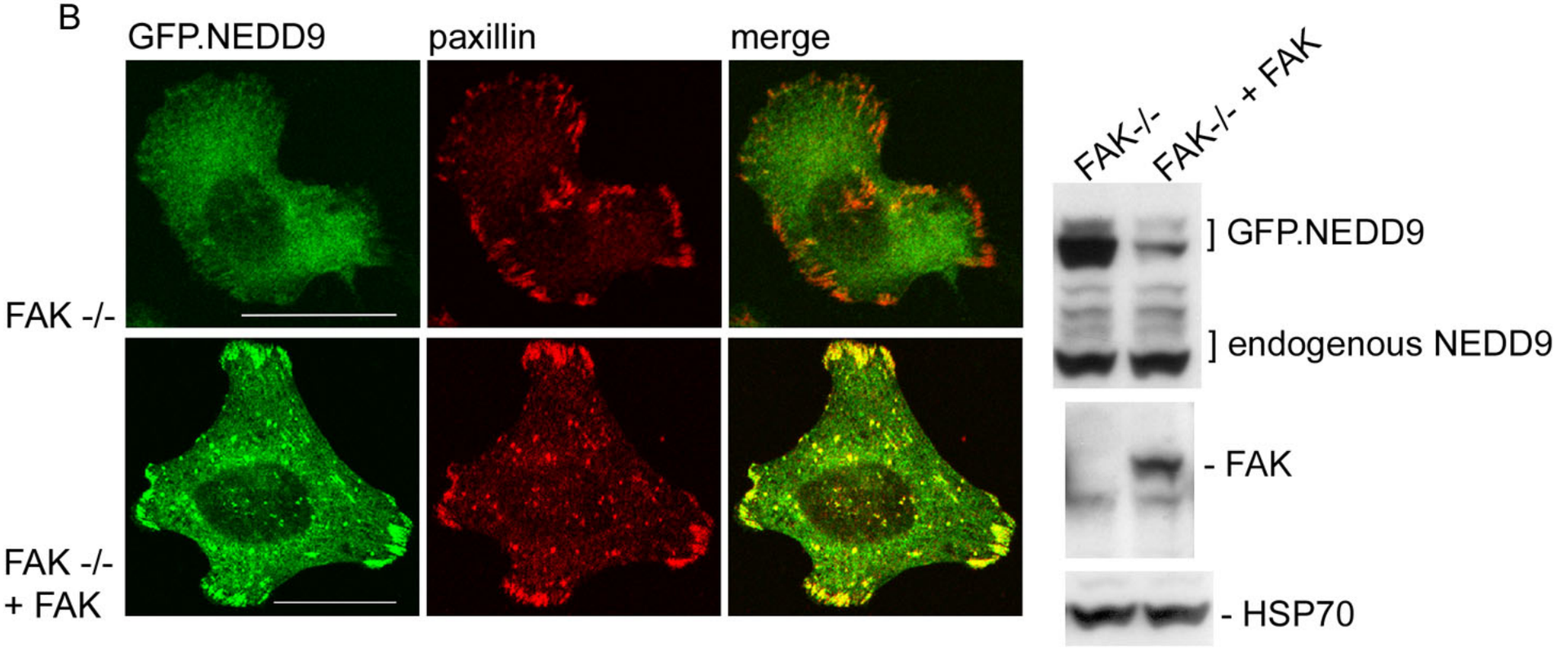

B
GFP.NEDD9
paxillin
merge
FAK -/-
FAK -/-
+ FAK
FAK-/-
FAK-/- + FAK
] GFP.NEDD9
] endogenous NEDD9
- FAK
- HSP70

FIGURE 6

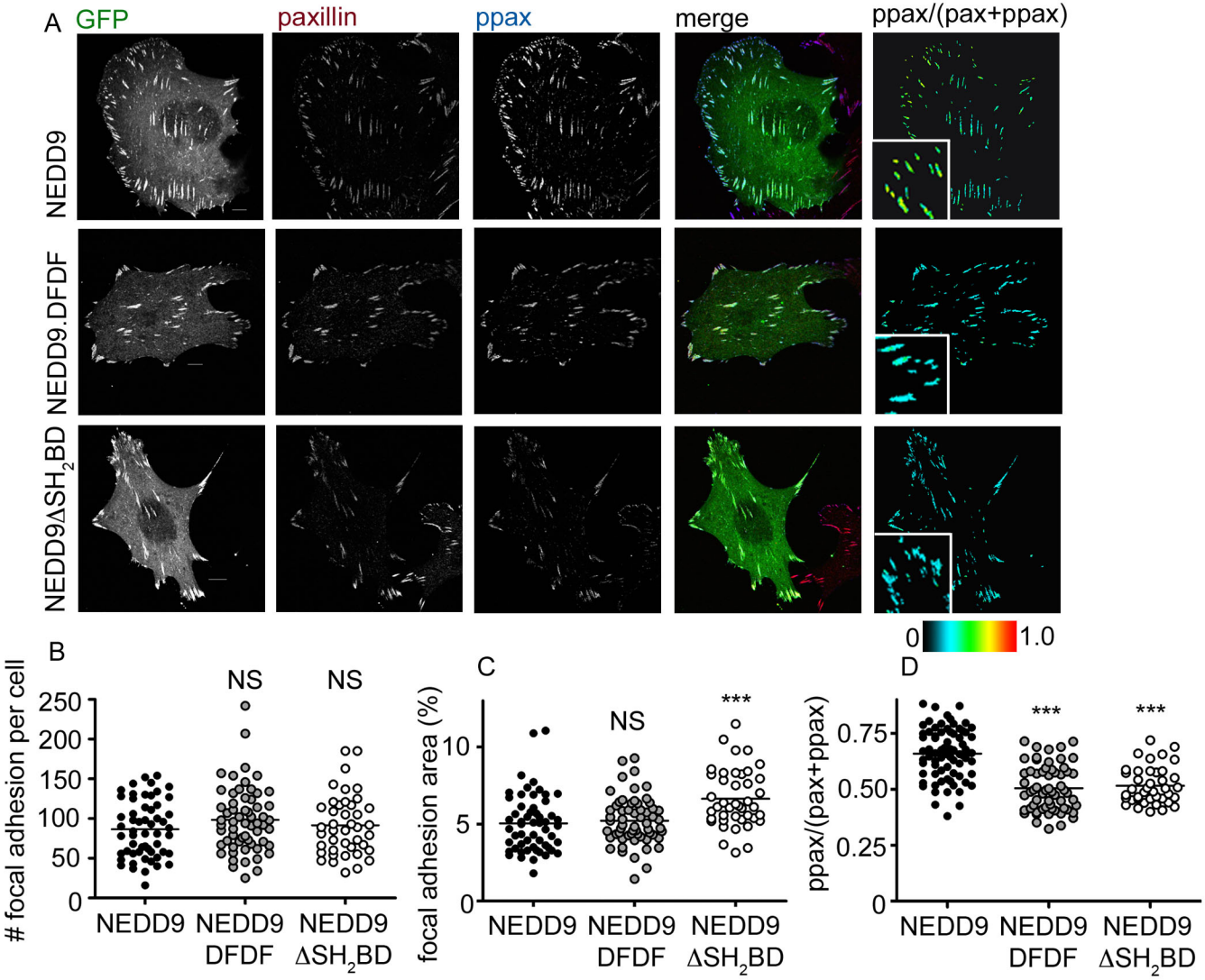
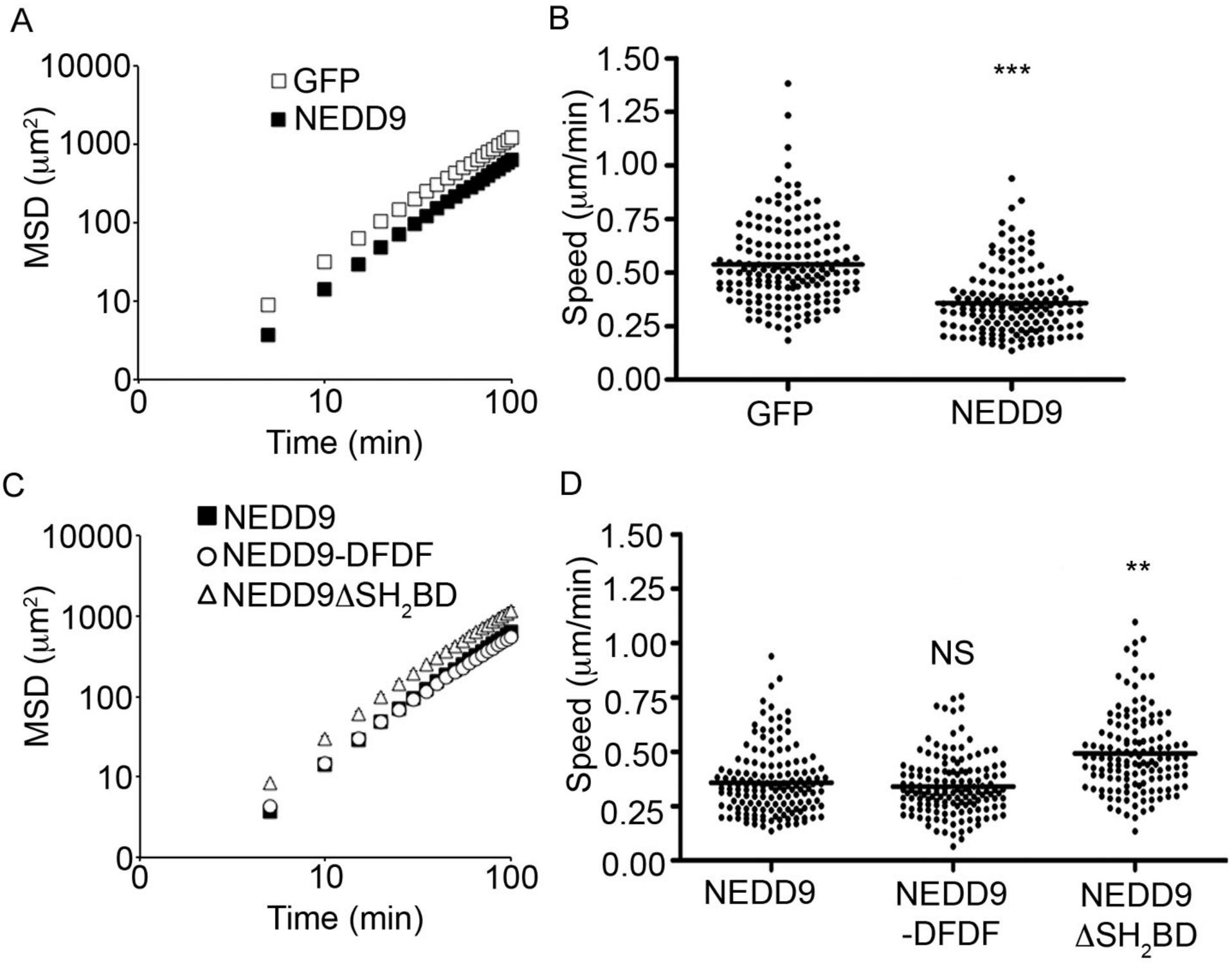

A
GFP
paxillin
ppax
merge
ppax/(pax+ppax)
NEDD9
NEDD9.DFDF
NEDD9ΔSH2BD
0
1.0
B
focal adhesion per cell
250
200
150
100
50
0
NS
NS
NEDD9
NEDD9 DFDF
NEDD9 ΔSH2BD
C
focal adhesion area (%)
10
5
0
NS
***
NEDD9
NEDD9 DFDF
NEDD9 ΔSH2BD
D
ppax/(pax+ppax)
0.75
0.50
0.25
0
***
***
NEDD9
NEDD9 DFDF
NEDD9 ΔSH2BD

FIGURE 7


A
10000
1000
100
10
0
MSD (μm²)
GFP
NEDD9
0
10
100
Time (min)
B
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Speed (μm/min)
***
GFP
NEDD9
C
NEDD9
NEDD9-DFDF
NEDD9ΔSH2BD
10000
1000
100
10
0
MSD (μm²)
0
10
100
Time (min)
D
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Speed (μm/min)
NS
**
NEDD9
NEDD9 -DFDF
NEDD9 ΔSH2BD