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Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins

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ABSTRACT

Cell migration requires the controlled disassembly of focal adhesions, but the underlying mechanisms remain poorly understood. Here, we show that adhesion turnover is mediated through dynamin- and clathrin-dependent endocytosis of activated β1 integrins. Consistent with this, clathrin and the clathrin adaptors AP-2 and disabled-2 (DAB2) distribute along with dynamin 2 to adhesion sites prior to adhesion disassembly. Moreover, knockdown of either dynamin 2 or both clathrin adaptors blocks β1 integrin internalization, leading to impaired focal adhesion disassembly and cell migration. Together, these results provide important insight into the mechanisms underlying adhesion disassembly and identify novel components of the disassembly pathway.

1. Introduction

Cell migration depends on the regulated formation and turnover of integrin-based focal adhesions [1,2]. New adhesion complexes form at the base of membrane protrusions at the cell front upon engagement of integrin receptors with extracellular matrix components, which leads to the clustering of integrins and the subsequent recruitment of scaffolding and signaling proteins [1,2]. The assembly of focal adhesions is necessary for the formation of a leading edge lamellipodium and the initiation of locomotion. However, the subsequent release of adhesion at the cell front and the trailing cell rear is equally as important for cell migration to continue [2,3]. Yet, while the mechanisms leading to focal adhesion formation have been studied extensively and are relatively well understood, the molecular mechanisms underlying focal adhesion disassembly remain largely unknown.

The protease calpain, microtubules, focal adhesion kinase (FAK) and the large GTPase dynamin were recently identified as critical mediators of focal adhesion disassembly during cell migration [4,5]. Calpain contributes to adhesion disassembly at the cell rear by cleaving specific focal adhesion proteins, including integrins and talin [4]. Furthermore, microtubules induce focal adhesion disassembly by directly targeting substrate adhesion complexes [5]. A key event in this process is the activation of FAK and its subsequent complex formation with dynamin 2 [3,5]. This is thought to induce the targeting of dynamin 2 to adhesion sites, thereby leading to their turnover [5]. The exact mechanism by which dynamin 2 promotes the turnover of adhesion complexes remains unknown. However, it is well-known that dynamin 2 is a key regulator of endocytosis [6], thus raising the question as to whether adhesion disassembly occurs by endocytosis of integrins.

Here, we present direct evidence in support of such a mechanism. We show that dynamin 2 mediates focal adhesion disassembly through endocytosis of a subpopulation of activated β1 integrins and we further identify clathrin and the two adaptor proteins DAB2 and AP-2 as novel components of the disassembly pathway.

2. Materials and methods

2.1. Antibodies, reagents, and plasmids

Antibodies were from Sigma (vinculin, zyxin), BD Transduction Laboratories (FAK, dynamin 2, disabled-2), Santa Cruz (Clathrin heavy chain; sc-6579), Affinity BioReagents (α-adaptin), Chemicon (human β1 integrin MAB1981), Abcam (human β1 integrin 12G10) and Jackson ImmunoResearch (Cy2-, Cy3-, and Cy5-conjugated IgGs). Rabbit polyclonal anti-dynamin 2 antibodies and plasmids containing GFP-dynamin 2(aa) and dynamin 2 mutant variants ($Dyn2^{PRD}$ or $Dyn2^{K44A}$) were a gift from Mark A. McNiven (Mayo Clinic, Rochester, MI).

2.2. Cell culture

The human fibrosarcoma HT1080 cell line (ATCC) was grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% calf serum, penicillin and streptomycin with 5% CO_2 at 37 °C.

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2.3. siRNA transfection and rescue

siGENOME SMARTpool reagents (dynamin 2, clathrin heavy chain, DAB2, scrambled siCont) were purchased from Dharmacon. siRNA duplexes targeting the AP-2 mu2 subunit were previously described [7]. siRNA pools were transfected at a final concentration of 100 nM (single duplexes: 25 nM) into 30–50% confluent HT1080 cells using Lipofectamine 2000 as recommended by the manufacturer. siRNA-treated cells were analyzed 48 h post-transfection. Efficacy of RNAi knockdown was confirmed by immunoblot analysis and typically resulted in a reduction of protein levels by 75–80%. For rescue experiments, siRNA-treated HT1080 cells were transfected 36 h after RNAi treatment with cDNAs encoding Flag- or GFP-tagged wild type or mutant dynamin 2 or empty vector and cells were assayed 12 h later as described. Transfection efficiency typically was 90% as judged by GFP fluorescence.

2.4. Immunofluorescence analysis

Immunofluorescence analysis was performed essentially as described [8].

2.5. Drug treatments

HT1080 cells were pre-incubated for 10–30 min at 37 °C in the presence of vehicle (DMSO), 60 μM monodansyl-cadaverine (MDC) or 10 μM filipin before fixation and immunostaining.

2.6. Focal adhesion disassembly

Focal adhesion disassembly assays were performed as described [5]. HT1080 cells were serum-starved for 12 h prior to treatment with 10 μM nocodazole for 1 h. Nocodazole was washed away and replaced with serum-free medium and, at

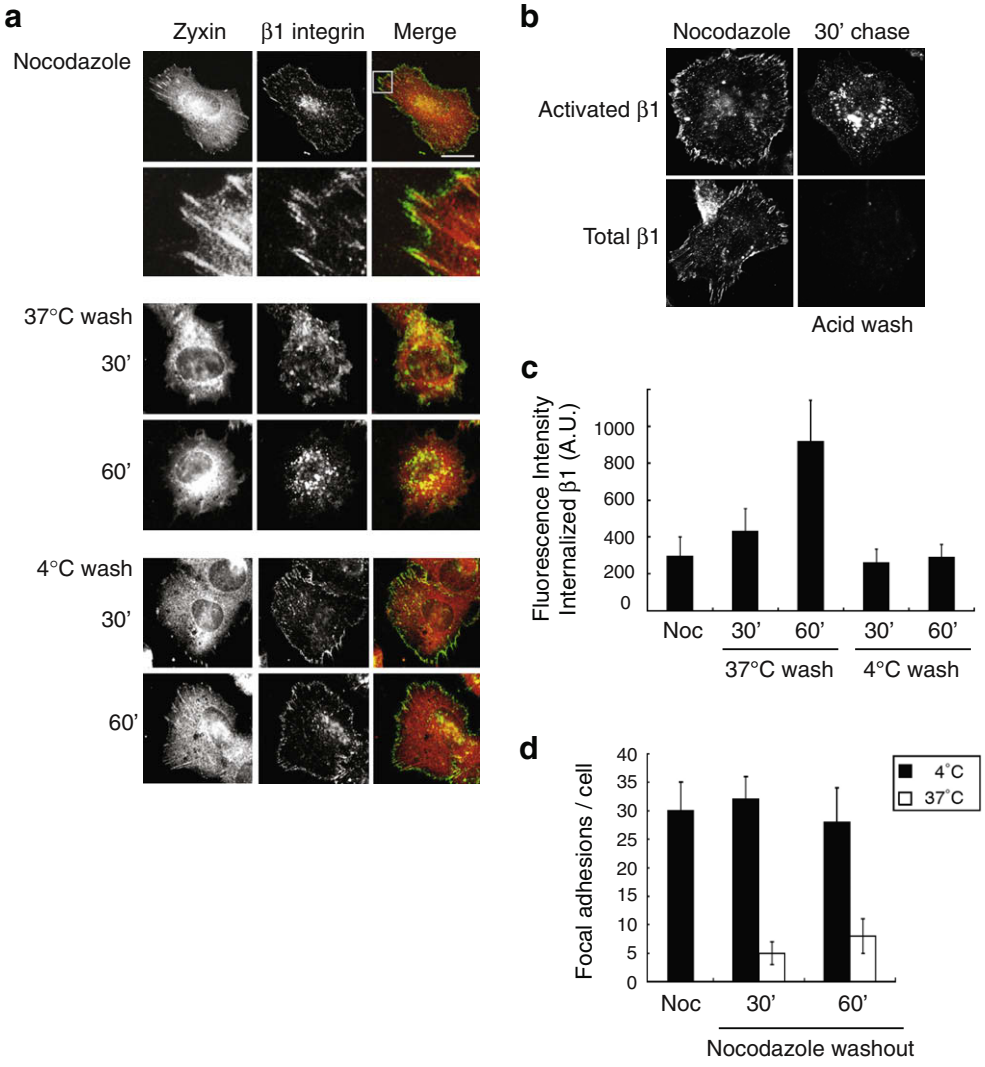

Fig. 1. β1 integrin internalization is necessary for focal adhesion disassembly. (a) Nocodazole-treated HT1080 cells were incubated with 12G10 antibody at 4 °C to label ligand-bound cell surface β1 integrin. After removal of unbound antibodies and nocodazole, cells were either immediately fixed and immunostained to visualize zyxin (red in merge) and antibody–β1 integrin complexes (green in merge) or were continued to incubate at 37 °C (middle panels) or 4 °C (bottom panels) for the indicated times before immunostaining. Shown are single confocal z-sections at the substratum-facing surface. Enlarged images of the boxed area are shown below the top panels. Scale bar, 10 μm. (b) Nocodazole-treated HT1080 cells were incubated at 37 °C for 20 min with antibodies that label either activated (12G10; activated) or total (MAB1298; total) β1 integrins on the cell surface. After nocodazole washout, cells were acid-stripped, fixed and immunostained to visualize integrin–antibody complexes. Shown are representative confocal z-sections at the substratum-facing surface. (c) Quantitative analysis of β1 integrin internalization from (a). Total immunofluorescence intensity of internalized antibody–integrin complexes was analyzed from z-sections to obtain a mean pixel intensity value (expressed in arbitrary units, A.U.). (d) Quantification of zyxin-positive focal adhesion numbers from (a). Values in (c) and (d) are means ± S.E.M. ($n = 3$).

the indicated times, cells were fixed in paraformaldehyde, permeabilized and immunostained with the indicated antibodies. The number of focal adhesions per cell from 100 to 150 cells in each group was then evaluated from photographs of zyxin staining. Data shown represent the means ± S.E.M. from three independent experiments.

2.7. Transwell migration

siRNA-treated HT1080 cells (1×10^5) were plated in the upper chamber of a Transwell chamber (8.0 μm pore size, BD Transduction Laboratories) and chemotaxis assays were performed with 10% FBS (vol/vol) medium added to the bottom chamber for 2 h at 37 °C. Non-migrating cells were removed from the top chamber, remaining cells were fixed in 3.7% formaldehyde/0.5% Triton X-100 in PBS, stained with DAPI to visualize nuclei, mounted, and cells from eight different areas were counted by fluorescence microscopy. Data from three independent experiments were collected.

2.8. Integrin internalization

β1 integrin endocytosis was measured using established procedures [9,10]. For pulse-chase experiments, serum-starved HT1080

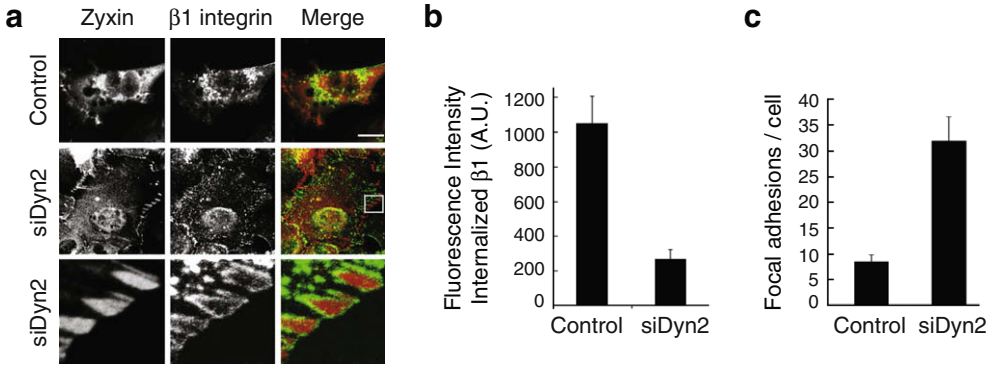

Fig. 2. Knockdown of dynamin 2 prevents the endocytosis of activated β1 integrins. (a) HT1080 cells, pretreated with control siRNA or siRNA targeting dynamin 2, were incubated with 12G10 integrin β1 antibody for 2 h at 37 °C to allow for internalization of β1 integrin–ligand complexes. Cells were then either directly fixed or acid-stripped prior to fixation to remove non-internalized surface antibody and cells were immunostained to visualize zyxin (red in merge) and activated β1 integrin–ligand complexes (green in merge). Shown are representative confocal z-sections at the substratum-facing surface. Lower panels show regions outlined by a box at higher magnification. Scale bar, 10 μm. (b) Quantitative analysis of internalized β1 integrin from (a). Total immunofluorescence intensity of internalized antibody–integrin complexes was measured as described in Fig. 1c. (c) Quantification of focal adhesion number from (a). Values shown in (b) and (c) are means ± S.E.M. ($n = 3$).

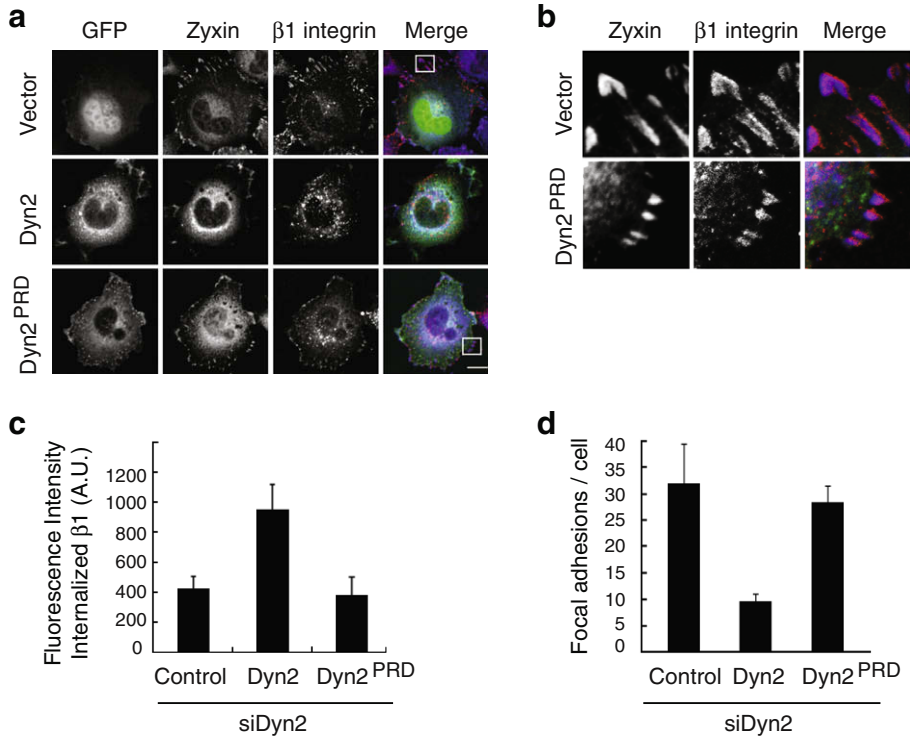

Fig. 3. Overexpression of dynamin 2, but not the Dyn2^{PRD} mutant in dynamin 2-depleted cells restores β1 integrin internalization and focal adhesion disassembly. (a) Dynamin 2 siRNA-treated cells were transfected with plasmids encoding GFP (Vector), GFP-tagged wild-type dynamin 2 (Dyn2), or the Dyn2^{PRD} mutant and were then incubated with 12G10 integrin β1 antibody for 2 h at 37 °C to allow for internalization of β1 integrin–ligand complexes. Cells were fixed, permeabilized and immunostained to visualize zyxin (blue in merge) and β1 integrin–antibody complexes (red in merge). GFP fluorescence was recorded directly (green in merge). Representative confocal z-sections at the substratum-facing surface are shown. Scale bar, 10 μm. (b) Regions outlined by boxes in (a) are shown at higher magnification. (c) Quantitative analysis of internalized β1 integrins. Immunofluorescence intensity of internalized β1 integrins was measured in the cell interior as described in Fig. 1c. (d) Quantification of focal adhesion number from (a). Values in (c) and (d) are means ± S.E.M. ($n = 3$).

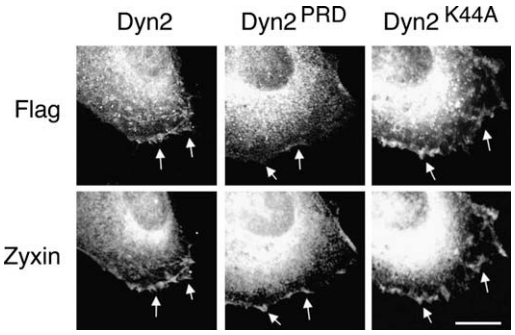

Fig. 4. Localization of dynamin 2 to focal adhesions requires its C-terminal PRD domain. HT1080 cells were transfected with Flag-tagged wild type dynamin 2 or the indicated Dyn2 mutant variants and were then treated with nocodazole, fixed and stained with anti-zyxin and anti-Flag antibodies. Only the $Dyn2^{PRD}$ mutant fails to accumulate within zyxin-positive adhesions (indicated by arrows). Scale bar, 10 μm.

cells were pretreated with nocodazole (10 μM, 1 h, 37 °C) and then incubated for 1 h at 4 °C with anti-human integrin β1–antibody 12G10, which preferentially recognizes the extracellular domain of activated human β1 integrin and enhances integrin activation. Unbound antibodies and nocodazole were washed away and the chase was performed in complete media containing 10% FBS at 37 °C or 4 °C. Alternatively, siRNA-treated cells were incubated at 37 °C with 12G10 antibody or with an antibody (MAB1981) that is non-activating and labels total β1 integrin. At the indicated times, cells were fixed in paraformaldehyde, permeabilized and immunostained using appropriate antibodies to visualize zyxin or β1 integrin–antibody complexes. Images were then acquired under identical parameters using a Zeiss LSM 510 META confocal microscope with 63× objective (1.4 oil, Zeiss). To quantify β1 integrin internalization, surface antibodies were removed prior to fixation by an acid rinse (0.5% acetic acid, 0.5 M NaCl, pH 3.0). Serial z-sections were obtained from individual cells every 0.5 μm and total fluorescent intensity per cell area for each confocal z-section was measured as described [10] to determine the mean level of internal β1 integrins. Data was collected from 30 to 40 cells per condition, sampled from three independent experiments.

2.9. Statistics

All statistics in this study were performed by Student *t*-test.

3. Results

3.1. Focal adhesion disassembly requires endocytosis of activated β1 integrins

To explore whether focal adhesion disassembly involves integrin endocytosis, we used a pulse-chase approach to monitor the

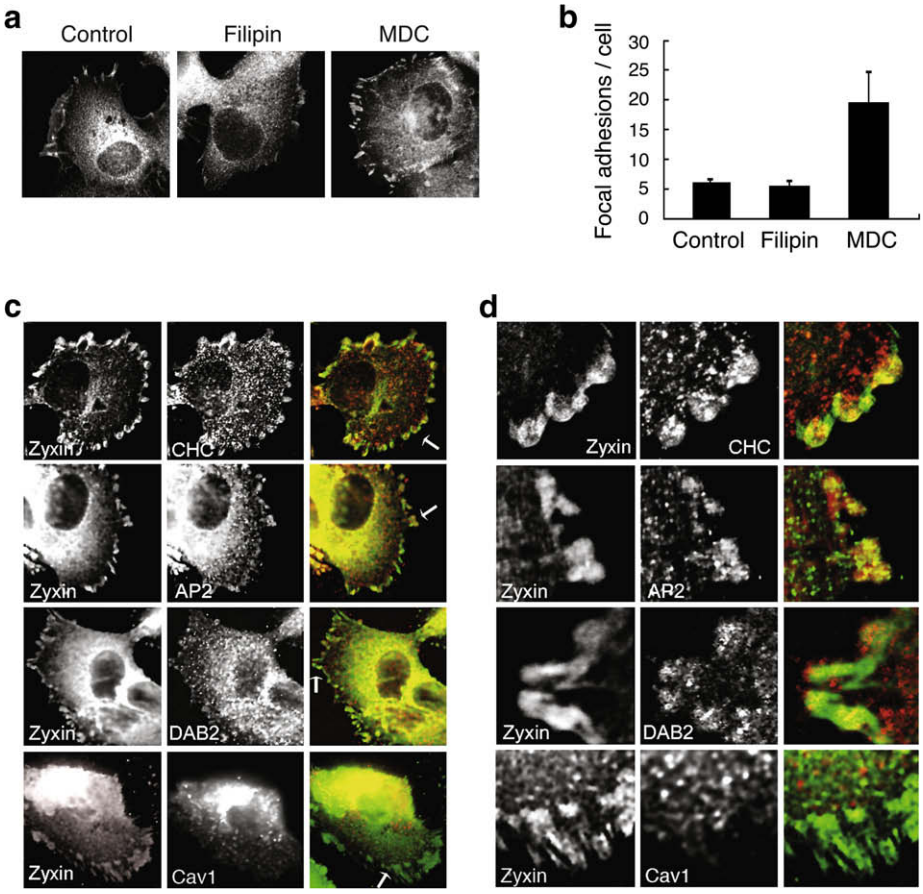

Fig. 5. Clathrin components distribute to focal adhesions before adhesion disassembly. (a) HT1080 cells were treated with vehicle (DMSO), filipin (10 μM) or MDC (60 μM) before fixation and staining for zyxin. (b) Quantification of zyxin-positive focal adhesion numbers from (a). Values are means ± S.E.M. from two independent experiments. (c) The localization of mRFP-tagged caveolin 1 (Cav1) was directly visualized in transfected nocodazole-treated HT1080 cells, whereas the localization of endogenous clathrin heavy chain (CHC), the AP-2 subunit α-adaptin, DAB2, and zyxin was analyzed by indirect immunofluorescence staining using specific antibodies. Representative examples of cells are shown. (d) A magnification of the indicated cell areas (arrows) in (c) is shown to highlight the localization of clathrin components (red in merge) within zyxin-containing (green in merge) adhesions.

fate of integrin β1, the predominant integrin subunit involved in cell spreading and migration in HT1080 fibrosarcoma cells [11]. To do so, cells were treated with the microtubule depolymerizing drug nocodazole to reversibly block focal adhesion disassembly [5]. Cells were then shifted to 4 °C, a non-permissive temperature for endocytosis, and incubated with an antibody (12G10) that specifically cross-reacts with the ligand-activated form of β1 integrin to label the surface pool of β1 clustered within focal adhesion sites. After washing steps to remove unbound antibody and nocodazole, cells were shifted to 37 °C for various periods of time to enable endocytosis before fixation and immunostaining. To quantitatively assess β1 integrin endocytosis, aliquots of cells were acid-stripped prior to fixation to remove surface-bound antibody. By employing these approaches, we found that activated β1 integrins were initially distributed within enlarged adhesions that accumulated around the cell periphery in HT1080 cells (Fig. 1a–c). However, within 30 min after shift to 37 °C, β1 integrin–antibody complexes redistributed from the cell surface to an intracellular pool and by 60 min were exclusively observed in perinuclear aggregates in over 95% of cells (Fig. 1a and c). Intriguingly, when the uptake of total cell surface β1 integrin was measured using a conformation-independent antibody, the internalization of bulk β1 integrin was found to be significantly delayed when compared to activated β1 integrin (Fig. 1b). Thus, only β1 integrins in their active conformation appear to be endocytosed during focal adhesion disassembly. Notably, the uptake of activated β1 integrins mirrored the disassembly of adhesion sites, which was simultaneously monitored by immunostaining of cells for zyxin, a marker for stable adhesions. Accordingly, focal adhesions accumulated during nocodazole treatment (Fig. 1a and d), but these adhesions completely disassembled in a synchronous manner in the entire cell population within 30 min after nocodazole washout (Fig. 1a and d). These data therefore reveal a good correlation between the rate of focal adhesion turnover and the rate of β1 integrin uptake. Indeed, when the internalization of β1 integrin–antibody complexes was inhibited by incubating cells at 4 °C during nocodazole washout (Fig. 1a and c), focal adhesion disassembly was blocked in greater than 90% of cells (Fig. 1d). Thus, the turnover of adhesion sites appears to depend on the endocytosis of a subpopulation of activated β1 integrins.

3.2. Dynamin 2 is necessary for the endocytosis of activated β1 integrins

Consistent with the idea that dynamin 2 mediates focal adhesion disassembly via integrin endocytosis, we found that dynamin 2 knockdown resulted in the retention of β1 integrins within zyxin-containing adhesions in 89% of cells (Fig. 2a–c). This defect could be rescued by the reintroduction of GFP-tagged wild type dynamin 2 (Fig. 3a–c). The same construct also corrected the focal adhesion phenotype of these cells (Fig. 3d). By contrast, a dynamin 2 mutant ($Dyn2^{PRD}$) that lacks the C-terminal proline-rich (PRD) domain necessary for the formation of dynamin–FAK complexes and focal adhesion disassembly [5] failed to suppress the endocytosis and focal adhesion defects associated with dynamin 2 knockdown (Fig. 3a–d). Importantly, unlike wild type dynamin 2 or a mutant variant defective in GTPase activity ($Dyn2^{K44A}$), the $Dyn2^{PRD}$ mutant failed to efficiently distribute to focal adhesions

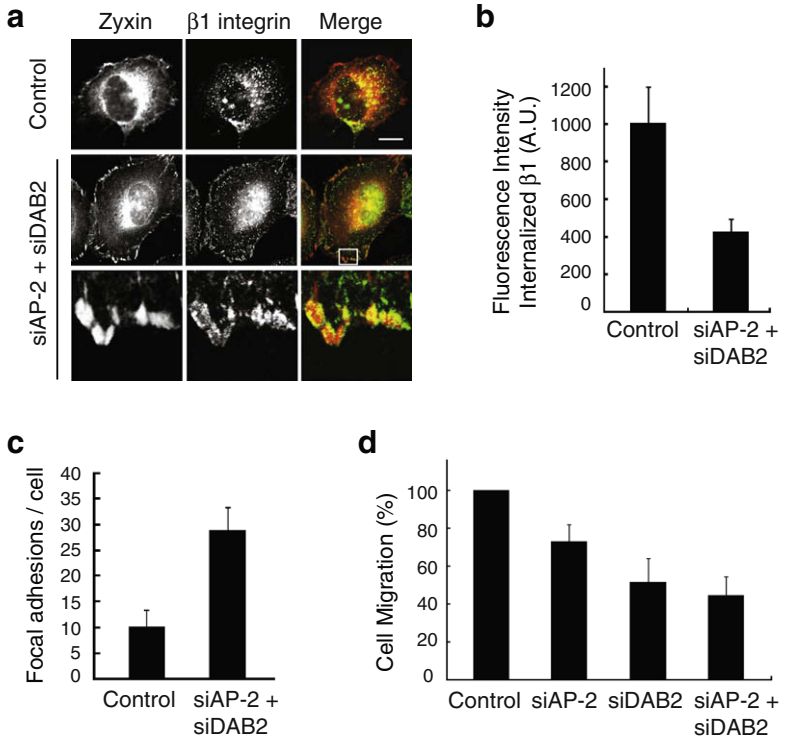

Fig. 6. Combined knockdown of AP-2 and DAB2 inhibits the endocytosis of activated β1 integrins and focal adhesion disassembly. (a) HT1080 cells, pretreated with control siRNA or combined siRNA targeting AP-2 and DAB2, were incubated with 12G10 integrin β1 antibody for 2 h at 37 °C to allow for internalization of β1 integrin–ligand complexes. Cells were then either directly fixed or acid-stripped prior to fixation to remove non-internalized surface antibody from cells and were then immunostained for zyxin (red in merge) and β1 integrin–ligand complexes (green in merge). Shown are representative confocal z-sections at the substratum-facing surface. Lower panels show enlarged images of the boxed area. Scale bar, 10 μm. (b) Quantitative analysis of internalized β1 integrins. Total immunofluorescence intensity of internalized antibody–integrin complexes was measured as described in Fig. 1c. (c) Quantification of focal adhesion number from (a). Values in (a–c) are means ± S.E.M. ($n = 3$). (d) Cells treated with the indicated siRNAs were serum-starved and cell migration across Transwell membranes was assayed in response to serum (10% FBS) present in the bottom chamber. The total number of cells passing through the membrane was determined. Results shown are expressed as the percentage of migrated cells relative to control cells, which was arbitrarily set to 100%.

prior to focal adhesion disassembly (Fig. 4), suggesting that complex formation between dynamin 2 and FAK induces the recruitment of dynamin 2 to adhesion sites to facilitate their disassembly by endocytosis.

3.3. Focal adhesion disassembly is a clathrin-dependent process

Dynamin 2 plays a central role in clathrin-dependent and -independent endocytosis [6]. To further delineate the pathway through which dynamin regulates adhesion disassembly, we performed experiments with compounds that selectively inhibit specific endocytosis routes. Treatment of cells with monodansyl-cadaverine (MDC), an inhibitor of clathrin-mediated endocytosis [12], markedly increased focal adhesion number and size (Fig. 5a and b), thus mimicking the effect of dynamin 2 knockdown. Conversely, treatment of cells with filipin, which blocks lipid raft-dependent internalization [12], did not affect focal adhesion number (Fig. 5a and b). Thus, focal adhesion disassembly may occur by a clathrin-dependent mechanism. This conclusion was corroborated by experiments showing that clathrin or the clathrin adaptor AP-2 became enriched within zyxin-containing focal adhesions that accumulated in nocodazole-treated cells (Fig. 5c and d), whereas a transiently expressed mRFP fusion to caveolin-1, a component of lipid raft-dependent internalization pathways [6], did not (Fig. 5c and d).

Moreover, when clathrin-dependent endocytosis was blocked by siRNA-mediated depletion of clathrin heavy chain, the internalization of activated β1 integrins was significantly decreased by 55–60% (data not shown). However, CHC knockdown failed to induce the accumulation of focal adhesions, presumably because the surface expression of β1 integrins was dramatically reduced upon CHC knockdown and the majority of β1 integrin became trapped in intracellular vesicles (data not shown). Thus, clathrin appears to also be necessary for the delivery or recycling of β1 integrins to the plasma membrane and their incorporation into focal adhesion complexes. To circumvent this complication, we depleted cells for the clathrin adaptor AP-2, which is predominantly involved in the formation of clathrin-coated vesicles at the plasma membrane [13]. Suppression of the AP-2 μ2 subunit resulted in a small but statistically significant reduction in β1 integrin uptake by ~20% (data not shown). While supportive of a role of AP-2 in focal adhesion disassembly, these findings suggested that AP-2 acts redundantly with another clathrin adaptor(s). A good candidate is the alternative clathrin adaptor DAB2, a member of a related family of cargo-specific adaptor proteins that bind clathrin and AP-2 [14,15]. DAB2 has been shown to functionally overlap with AP-2 [14,15] and consistent with the idea that DAB2 and AP-2 act redundantly to control the uptake of activated β1 integrins during focal adhesion disassembly, endogenous DAB2 became enriched at zyxin-positive adhesions in nocodazole-treated cells (Fig. 5c and d). Moreover, the combined knockdown of DAB2 and AP-2 markedly attenuated β1 integrin endocytosis and focal adhesion disassembly (Fig. 6a–c), and also produced a significant inhibitory effect on cell migration (Fig. 6d), which is in accord with evidence that clathrin-dependent internalization of β1 integrins plays a role in cell motility [10]. By comparison, depletion of DAB2 alone failed to produce a focal adhesion phenotype and caused less pronounced effects on β1 integrin uptake, adhesion disassembly, and cell migration (Fig. 6d and data not shown). Collectively, these data identify DAB2 as a critical regulator of focal adhesion disassembly and

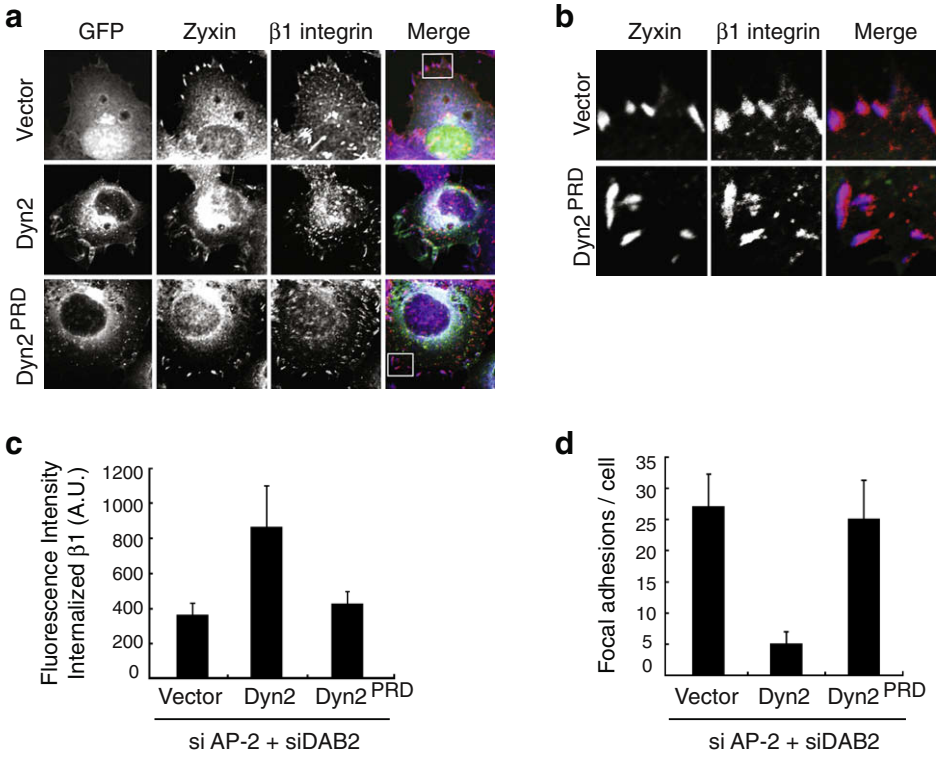

Fig. 7. Overexpression of dynamin 2, but not the Dyn2^{PRD} mutant in AP-2/DAB2-depleted cells restores β1 integrin internalization and focal adhesion disassembly. (a) HT1080 cells treated with AP-2 and DAB2 siRNAs were transfected with plasmids encoding GFP (Vector), GFP-tagged wild-type dynamin 2 (Dyn2), or the Dyn2^{PRD} mutant and were then incubated with 12G10 integrin β1 antibody for 2 h at 37 °C to allow for internalization of integrin–antibody complexes. Cells were washed, fixed, and immunostained to visualize zyxin (blue in merge) and β1 integrin–antibody complexes (red in merge). GFP fluorescence was recorded directly (green in merge). Representative confocal z-sections at the substratum-facing surface are shown. (b) Regions outlined by boxes in (a) are shown at higher magnification. (c) Quantitative analysis of internalized β1 integrins. Total immunofluorescence intensity of internalized antibody–integrin complexes was measured as described in Fig. 1c. (d) Quantification of focal adhesion number from (a). Values in (c) and (d) are means ± S.E.M. (n = 3).

show that DAB2 acts together with the classic clathrin adaptor AP-2 to promote the endocytosis of activated β1 integrins.

Interestingly, the defects associated with combined AP-2 and DAB2 knockdown were efficiently suppressed by overexpression of dynamin 2, but not by the $Dyn2^{PRD}$ mutant (Fig. 7). This effect was completely reverted by treatment of the dynamin 2-overexpressing cells with MDC (data not shown), thus excluding the possibility that dynamin 2 acts through a compensatory endocytic pathway. Therefore, dynamin 2 may enhance the scission of residual clathrin-coated vesicles that form in AP-2/DAB2 knockdown cells, a role that is consistent with the finding that dynamin 2 controls a rate-limiting step in endocytosis [16]. Collectively, these data suggest that dynamin 2 acts together with DAB2 and AP-2 to promote focal adhesion disassembly and further emphasize the critical role of dynamin 2 in this process.

4. Discussion

There is mounting evidence that the cell surface distribution of integrins in migrating cells is controlled by cycles of endo- and exocytosis [17]. This is thought to play a central role in cell migration by removing integrins from the plasma membrane at the base of protrusions and at retracting edges so that they can be recycled back to the leading edge to support the formation of new adhesions [17]. However, whether integrin endocytosis also contributes to focal adhesion turnover remained unknown. The findings presented here provide the first direct evidence in support of such a hypothesis and show that focal adhesion disassembly occurs through dynamin- and clathrin-mediated endocytosis of a subpopulation of activated β1 integrins.

Our data further identify the alternative clathrin adaptor DAB2 as a novel regulator of focal adhesion disassembly that acts in conjunction with AP-2 to mediate integrin β1 internalization. These findings are consistent with a recent report showing that DAB2 is required for cell spreading [18]. DAB2 binds NPXY motifs, which serve as endocytic sorting signals found in the cytosolic tails of cargo proteins [19,20], and induces clathrin-mediated endocytosis of its cargo by simultaneously binding to clathrin or AP-2 [14,21]. NPXY motifs also directly recruit AP-2, leading to clathrin assembly [19,22]. Interestingly, the β1 integrin cytoplasmic domain contains two NPXY motifs [23]. These motifs may therefore serve as recognition sequences for DAB2 and AP-2, which then engage with each other and with clathrin to mediate β1 integrin endocytosis during adhesion disassembly. Consistent with such a hypothesis, the mutation of NPXY motifs in β1 integrin results in increased focal contacts and impaired cell motility [24]. Surprisingly, there is at present no direct evidence supporting a role of NPXY motifs in integrin endocytosis [25]. However, earlier studies addressing the role of NPXY motifs in β1 integrin uptake have focused on bulk β1 integrin endocytosis, whereas our data suggest that the activation of integrins is a prerequisite for their engagement with the endocytic machinery. Thus, the role of NPXY motifs in β1 integrin endocytosis should be revisited focusing on activated β1 integrin.

Interestingly, DAB2 is a distant relative of autosomal recessive hypercholesterolemia (ARH) [26] and of numb, which has been recently shown to control the endocytosis of integrins in conjunction with AP-2 during directed cell migration of epithelial cells [10]. Thus, future studies should focus not only on the precise mechanisms whereby integrins engage adaptor proteins, but also on determining whether numb and/or ARH play a role in focal adhesion disassembly. The studies presented here should provide a useful avenue to investigate these issues.

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