

# EHD1 regulates $\beta 1$ integrin endosomal transport: effects on focal adhesions, cell spreading and migration

Marko Jović<sup>1</sup>, Naava Naslavsky<sup>1</sup>, Debora Rapaport<sup>2</sup>, Mia Horowitz<sup>2</sup> and Steve Caplan<sup>1,\*</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology and Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE 68198-5870, USA

<sup>2</sup>Department of Cell Research and Immunology, Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel

\*Author for correspondence (e-mail: scaplan@unmc.edu)

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## Summary

$\beta 1$  integrins bind to the extracellular matrix and stimulate signaling pathways leading to crucial cellular functions, including proliferation, apoptosis, cell spreading and migration. Consequently, control of  $\beta 1$  integrin function depends upon its subcellular localization, and recent studies have begun to unravel the complex regulatory mechanisms involved in integrin trafficking. We report that the C-terminal Eps15-homology (EH) domain-containing protein EHD1 plays an important role in regulating  $\beta 1$  integrin transport. Initially, we demonstrated that RNAi-knockdown of *Ehd1* results in impaired recycling of  $\beta 1$  integrins and their accumulation in a transferrin-containing endocytic recycling compartment. Mouse embryonic fibroblast (MEF) cells derived from EHD1-knockout mice (*Ehd1*<sup>-/-</sup> MEF) exhibited lower overall levels

of  $\beta 1$  integrins on the plasma membrane, but higher cell-surface-expressed activated  $\beta 1$  integrins, and larger, more prominent focal adhesions resulting from slower kinetics of focal adhesion disassembly. In addition, both migration and cell spreading on fibronectin were impaired in *Ehd1*<sup>-/-</sup> MEF cells, and these defects could be similarly induced by EHD1-RNAi treatment of normal *Ehd1*<sup>+/+</sup> MEF cells. They could also be rescued by transfection of wild-type EHD1 into *Ehd1*<sup>-/-</sup> MEF cells. Our data support a role for EHD1 in  $\beta 1$  integrin recycling, and demonstrate a requirement for EHD1 in integrin-mediated downstream functions.

Key words: EHD1,  $\beta 1$  integrin, Recycling, Focal adhesions, Motility, Cell spreading

## Introduction

Integrins are the major eukaryote receptors for cell adhesion to the extracellular matrix (ECM). Upon ligand binding, heterodimeric  $\alpha/\beta$  integrin receptors cluster to cytoskeleton-linked focal adhesion complexes and induce the recruitment of kinases and adaptor proteins leading to the transduction of signals to the cell interior (reviewed by Hynes, 2002). The binding of  $\beta 1$  integrins to the ECM, including laminin, collagen, vitronectin (VN) and fibronectin (FN), stimulates a variety of cellular signaling pathways that may lead to gene expression, proliferation, apoptosis, cell survival, cell spreading, migration, invasion, metastasis and angiogenesis (reviewed by Varner and Cheresch, 1996). Integrins can also transduce inside-out signals, whereby intracellular signaling influences the affinity of integrins for their ECM ligands (Hynes, 2002).

To function and transmit signals,  $\beta 1$  integrins must localize to the plasma membrane and be able to relocate to focal adhesions upon ligand binding. Accordingly, the internalization process is crucial in regulating the levels of integrins localized to the plasma membrane and in maintaining functionality of integrin-mediated downstream effects. Whereas some integrins contain a cytosolic NITY motif conforming to the classical NxxY internalization motif found on receptors internalized through AP-2/clathrin-coated pits,

most integrins (including  $\beta 1$  integrin) appear to be internalized in a clathrin-independent manner (Altankov and Grinnell, 1995; Ylanne et al., 1995) that depends on the small GTPase Arf6 (Brown et al., 2001). Indeed, integrin internalization is regulated by protein kinase C- $\alpha$  (Ng et al., 1999) and dynamin (Ezratty et al., 2005; Ng et al., 1999), and several studies provide evidence that this process occurs either through caveolae and/or through detergent-resistant lipid microdomains (Upla et al., 2004; Wary et al., 1996; Wary et al., 1998).

Once internalized, integrin receptors return to the plasma membrane by a recycling route through the endocytic pathway (reviewed by Caswell and Norman, 2006; Jones et al., 2006; Pellinen and Ivaska, 2006). Certain integrin receptors, such as  $\alpha v \beta 3$ , are recycled rapidly to the plasma membrane through a short loop, whereas  $\alpha 5 \beta 1$  returns to the cell surface through a transitory recycling compartment (long-loop) (Roberts et al., 2001). Therefore, influencing the rate of integrin receptor recycling through these pathways is another crucial way in which integrin function may be modulated, and it has been widely accepted that the recycling of internalized integrins to the leading lamellae of a migrating cell affects its motility (Bretscher, 1996). Recent studies demonstrate that protein kinase C (Becker and Hannun, 2003; Ivaska et al., 2005; Ng et al., 1999) and protein kinase D1 (Woods et al., 2004) play

important roles in controlling  $\beta 1$  integrin recycling to the plasma membrane. In addition, the recycling of integrins depends upon many of the same endocytic regulatory proteins that control the transport of other internalized receptors. In particular, the GTP-binding proteins Rab4 (Roberts et al., 2001), Rab11 (Powelka et al., 2004) and Arf6 (Powelka et al., 2004), and recently Rab21 and Rab5 (Pellinen et al., 2006), have been implicated in integrin recycling.

Based on these studies, it is likely that other regulators of endocytic recycling and proteins that physically and/or functionally interact with Rab-family proteins and Arf6 play a role in the regulation of integrin trafficking. One family of proteins that fits this description is the C-terminal EHD protein family (reviewed by Miliaras and Wendland, 2004; Montesinos et al., 2005; Naslavsky and Caplan, 2005), which has been implicated in endocytic transport and recycling of a variety of receptors (Caplan et al., 2002; Guilherme et al., 2004; Lin et al., 2001; Park et al., 2004; Picciano et al., 2003; Rotem-Yehudar et al., 2001; Shao et al., 2002). In mammals the four highly homologous paralogs (EHD1, EHD2, EHD3 and EHD4) all contain nucleotide-binding motifs (Lee et al., 2005; Naslavsky et al., 2006), a central coiled-coil involved in homo- and/or hetero-oligomerization (Galperin et al., 2002; Lee et al., 2005; Naslavsky et al., 2006), and a conserved C-terminal Eps15-homology (EH)-domain that binds to proteins containing the tripeptide asparagine-proline-phenylalanine (NPF) (Braun et al., 2005; Guilherme et al., 2004; Naslavsky et al., 2004; Naslavsky et al., 2006; Rotem-Yehudar et al., 2001; Xu et al., 2004).

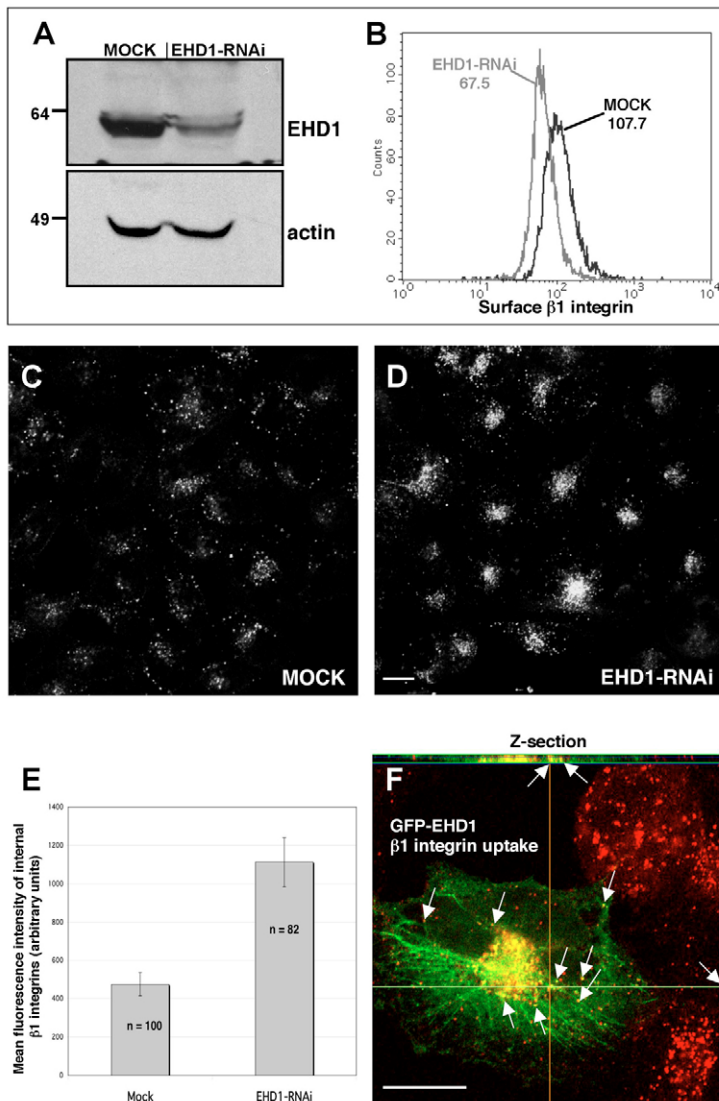
Recent studies have shown that EHD proteins functionally interact with Arf6 and coordinate recycling events with Rab4 and Rab11. For example, EHD1 colocalizes with and is regulated by the guanine nucleotide-binding status of Arf6 (Caplan et al., 2002). Moreover, EHD4 binds to the cell fate determinant, Numb, whose subcellular localization also depends upon Arf6 nucleotide binding (Smith et al., 2004). In addition, recent studies demonstrate a direct interaction between EHD proteins and effectors of Rab4 and Rab11. The divalent Rab4/Rab5 effector Rabenosyn-5 interacts with EHD1 and its paralogs and plays a role in transport from early endosomes to the endocytic recycling compartment (Naslavsky et al., 2004). More recently, an interaction between the Rab11-effector Rab11-FIP2 and both EHD1 and EHD3 was identified, highlighting a coordinated recycling mechanism jointly controlled by Rab11 and EHD proteins (Naslavsky et al., 2006). Given the involvement of Rab-family proteins and Arf6 in integrin-mediated transport and recycling, we now demonstrate that EHD1 plays an essential role in regulating the trafficking of  $\beta 1$  integrins and their downstream functions.

## Results

To assess whether EHD1 affects the subcellular itinerary of  $\beta 1$  integrins, we used an RNAi approach in HeLa cells, effectively reducing EHD1 levels by 80–90% (Fig. 1A) (Naslavsky et al., 2004). To follow  $\beta 1$  integrin trafficking, we used a modified version of the assay developed by Powelka et al. using the 12G10 anti- $\beta 1$  integrin antibody to label surface  $\beta 1$  integrins (Powelka et al., 2004). Cells were then allowed to internalize integrin-antibody complexes at 37°C for 2 hours (pulse), followed by a 2-hour incubation in the absence of antibody (chase) to monitor the distribution of internalized  $\beta 1$  integrins

over time. Internal  $\beta 1$  integrins in mock-treated cells were mainly dispersed in peripheral vesicles (Fig. 1C). However, cells treated with EHD1-RNAi displayed an accumulation of  $\beta 1$  integrin-containing vesicles in the perinuclear region (Fig. 1D). Quantification of the level of accumulated internal  $\beta 1$  integrin showed a greater than twofold increase in EHD1-RNAi-treated cells (Fig. 1E), suggesting a delay in integrin recycling and accumulation at the endocytic recycling compartment (ERC). Since it has been demonstrated that certain  $\beta 1$  integrin heterodimers recycle to the plasma membrane (reviewed by Bretscher, 1992; Caswell and Norman, 2006), we hypothesized that loss of EHD1 might decrease the rate of  $\beta 1$  integrin recycling to the plasma membrane. To this aim, mock- and EHD1-RNAi-treated HeLa cells were subjected to flow cytometry-based recycling assays to compare levels of  $\beta 1$  integrins that have returned to the plasma membrane. Non-internalized anti- $\beta 1$  integrin antibodies were stripped off the plasma membrane after the pulse by a brief acid rinse, a treatment that has been commonly used to follow trafficking of endogenous and chimeric proteins without interfering with the kinetics of their itinerary (Hemar et al., 1995). After a 2-hour chase at 37°C, antibody-integrin complexes that recycled back to the plasma membrane were detected with secondary antibodies under non-permeabilizing conditions. As shown (Fig. 1B), reduction of EHD1 expression typically decreased the levels of recycled  $\beta 1$  integrins localized to the plasma membrane by ~30%. To assess whether  $\beta 1$  integrins traffick through EHD1-containing membranes, we pulsed GFP-EHD1-transfected HeLa cells with antibodies against  $\beta 1$  integrins (Fig. 1F). As demonstrated in the representative 0.4  $\mu\text{m}$  z-section, the EHD1 structures tend to extend radially from the perinuclear recycling compartment region, and it was possible to distinguish individual vesicular membranes in three-dimensions containing both EHD1 and  $\beta 1$  integrins (see crosshairs and arrows), suggesting that  $\beta 1$  integrins indeed traverse an EHD1-regulated recycling pathway.

Previous studies have shown that either dominant-negative EHD1 (Lin et al., 2001) or EHD1-RNAi-knockdown (Naslavsky et al., 2004) cause accumulation of transferrin receptor at the ERC. To determine whether  $\beta 1$  integrins are similarly affected, we simultaneously compared the localization of internalized transferrin and  $\beta 1$  integrins in mock- and EHD1-RNAi-treated HeLa cells (Fig. 2). As expected, in mock-treated cells following the chase, transferrin was dispersed in small vesicles with only moderate levels of accumulation remaining at the ERC (Fig. 2A). The distribution of  $\beta 1$  integrins in these cells was also primarily a dispersed pattern with small punctate structures, showing partial overlap with transferrin-containing vesicles (Fig. 2A–C; arrows). However, EHD1-RNAi altered the subcellular distribution of internalized transferrin, inducing its accumulation at the ERC (Fig. 2D). In these same EHD1-RNAi-treated cells after chase,  $\beta 1$  integrins were also partially localized to a compact perinuclear region that showed partial overlap with the transferrin-containing structures at the ERC (Fig. 2D–F; arrows). Three-dimensional z-sections were obtained every 0.4  $\mu\text{m}$  (sample sections shown in Fig. 2C,F), and the crosshairs and arrows illustrate that 10–20% of the transferrin and  $\beta 1$  integrins are colocalized on common structures within the ERC. Collectively, these data suggest that in HeLa cells EHD1



**Fig. 1.** EHD1-knockdown affects  $\beta 1$  integrin transport and localization in HeLa cells. HeLa cells on plates (A,B) or coverslips (C,D,F) were mock-treated (A,B,C,F) or treated with EHD1-RNAi (A,B,D) for 48 hours. RNAi efficacy was determined following sample calibration for protein content, and immunoblotting for EHD1 (A; top panel) or actin control (A; bottom panel). Recycling of anti- $\beta 1$ -integrin- $\beta 1$ -integrin complexes to the plasma membrane in HeLa cells was quantitatively measured by a flow cytometry recycling assay using mouse anti-human 12G10 antibodies that preferentially recognize ligand-bound  $\beta 1$  integrins (MCA2028; Serotec) (B). The numbers denoted in the graph represent mean levels of  $\beta 1$  integrin fluorescence (from 10,000 cells) reappearing at the cell surface because of recycling. A similar pulse-strip-chase experiment was done in HeLa cells on coverslips to follow the internalization and subcellular distribution of  $\beta 1$  integrins upon EHD1-RNAi treatment (C compared with D). LSM 5 Pascale software was used to quantitatively determine the mean level of remaining, non-recycled internal  $\beta 1$  integrins by measuring the mean level of fluorescence per cell (E). Mock cells ( $n=100$ ) yielded a mean of 475 with a standard deviation of 121, whereas EHD1-RNAi-treated cells ( $n=82$ ) yielded a mean of 1112 with a standard deviation of 256. One-tailed Student's *t*-tests showed significance at  $P<0.0001$ . HeLa cells transfected with GFP-EHD1 were pulsed for 1 hour with 12G10 anti- $\beta 1$  integrin antibodies and visualized by confocal microscopy (F). Serial *z*-sections were obtained every 0.4  $\mu\text{m}$ , and the crosshairs and arrows depict common membrane structures on a representative micrograph containing both EHD1 (green) and  $\beta 1$  integrins (red). Bar, 10  $\mu\text{m}$ .

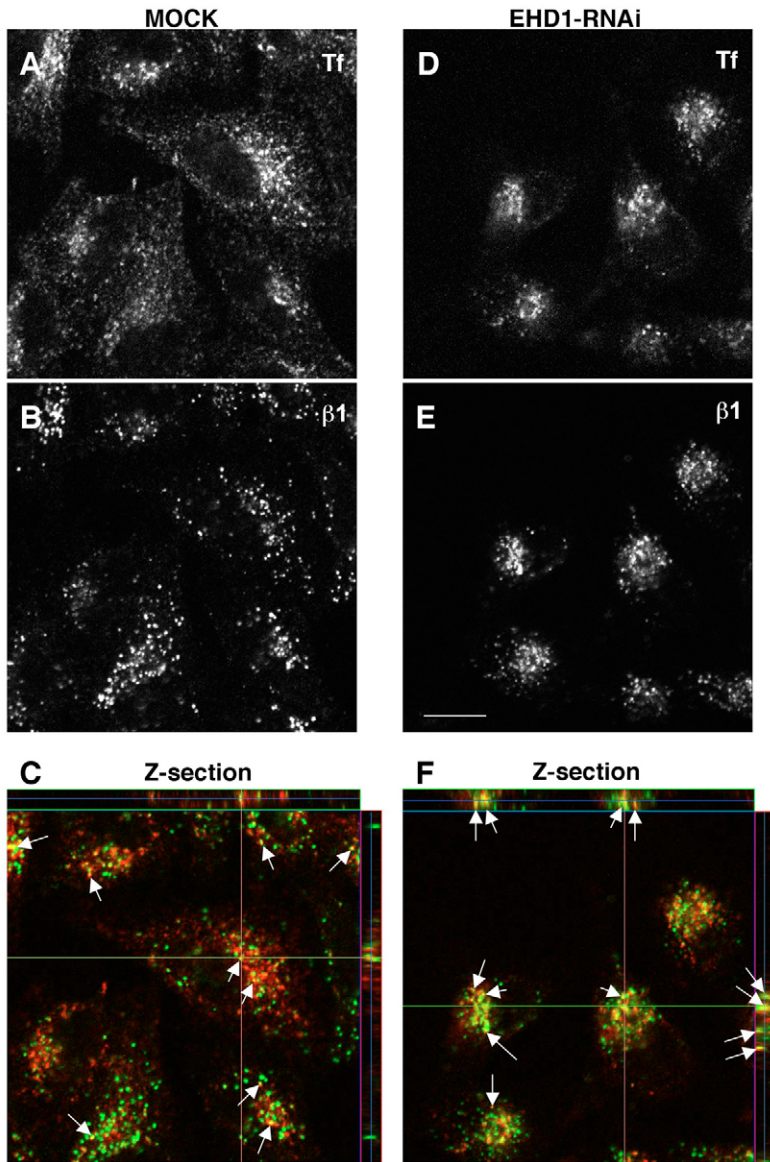
regulates the trafficking of internalized  $\beta 1$  integrins, possibly by controlling its exit from the ERC.

Integrins have been extensively studied in fibroblasts, and we next aimed to determine whether the loss of EHD1 in fibroblasts affected  $\beta 1$  integrin-mediated cellular functions. Accordingly, we first assessed the role of EHD1 in regulating  $\beta 1$  integrin trafficking in mouse embryonic fibroblast (MEF) cells derived from EHD1-knockout mice (*Ehd1*<sup>-/-</sup> MEF) (Rapaport et al., 2006). Both the normal *Ehd1*<sup>+/+</sup> MEF and *Ehd1*<sup>-/-</sup> MEF cells were subjected to fluorescence pulse-chase studies using 9EG7 anti- $\beta 1$  integrin antibodies. In these experiments, the level of fluorescence visualized within the cells represents  $\beta 1$  integrins that have not recycled to the plasma membrane. As demonstrated in Fig. 3, under these conditions  $\beta 1$  integrins are internalized slowly and after a 1-hour pulse, much of the signal is still detected on the plasma membrane in both *Ehd1*<sup>+/+</sup> MEF and *Ehd1*<sup>-/-</sup> MEF cells (Fig. 3A,B). Following a chase of 1 hour, the level of  $\beta 1$  integrins in *Ehd1*<sup>+/+</sup> MEF and *Ehd1*<sup>-/-</sup> MEF cells is similar (Fig. 3C,D), but in many of the *Ehd1*<sup>-/-</sup> MEF cells some accumulation is already noticeable in the ERC region (Fig. 3D). After 2 hours of chase, when compared with the *Ehd1*<sup>+/+</sup> MEF cells, there is

a higher level of accumulation observed in the *Ehd1*<sup>-/-</sup> MEF cells, particularly at the ERC (Fig. 3E,F; arrows). Upon 4 hours of chase, the *Ehd1*<sup>+/+</sup> MEF cells are mostly devoid of fluorescence, indicating that  $\beta 1$  integrin recycling is essentially complete (Fig. 3G). However, at this time point *Ehd1*<sup>-/-</sup> MEF cells still retain detectable levels of  $\beta 1$  integrins within the cells (Fig. 3H). Indeed, quantification of  $\beta 1$  integrins retained in *Ehd1*<sup>+/+</sup> MEF and *Ehd1*<sup>-/-</sup> MEF cells indicates an almost twofold difference in recycled  $\beta 1$  integrins at this time point, resulting from loss of EHD1 expression (Fig. 3I).

Since the four mammalian C-terminal EHD proteins are highly homologous and may interact with one another in protein complexes, we aimed to determine whether the other EHDs were also affected by loss of EHD1 expression, or whether the functional defects could be primarily attributed to loss of EHD1. As demonstrated in Fig. 3J, despite the loss of EHD1 expression in the *Ehd1*<sup>-/-</sup> MEF cells, the levels of EHD2 and EHD4 were similar in both *Ehd1*<sup>-/-</sup> MEF and normal MEF cells. Although it was difficult to assess changes in EHD3 (owing to a combination of low expression levels of EHD3 in MEF cells and/or the inability of the anti-human EHD3 antibody to efficiently react with mouse EHD3), loss of EHD1





**Fig. 2.** Impaired recycling in the absence of EHD1 leads to accumulation of  $\beta 1$  integrin in the endosomal recycling compartment. The intracellular localization of labeled transferrin and  $\beta 1$  integrins was compared using pulse-chase analysis in HeLa cells treated for 48 hours with mock-RNAi (A–C) or EHD1-RNAi (D–F). Cells were pulsed with 12G10 antibody that preferentially recognizes ligand-bound human  $\beta 1$  integrin (MCA2028; Serotec) for 2 hours, acid-stripped to remove non-internalized antibodies and chased for 30 minutes at 37°C. During this chase, transferrin–Alexa-Fluor-568 (Tf-568) was added for 15 minutes, and the Tf-568 was then chased in complete media for the remaining 15 minutes prior to fixation. Permeabilized cells were incubated with Alexa-Fluor-488-conjugated donkey anti-mouse antibody and analyzed by z-sectioning; confocal images were obtained every 0.4  $\mu\text{m}$ . The representative images show the same z-sections through mock- and RNAi-treated cells, and crosshairs show colocalizations on x- and y-axes. Arrows highlight specific vesicular membrane structures containing both transferrin and  $\beta 1$  integrins. Bar, 10  $\mu\text{m}$ .

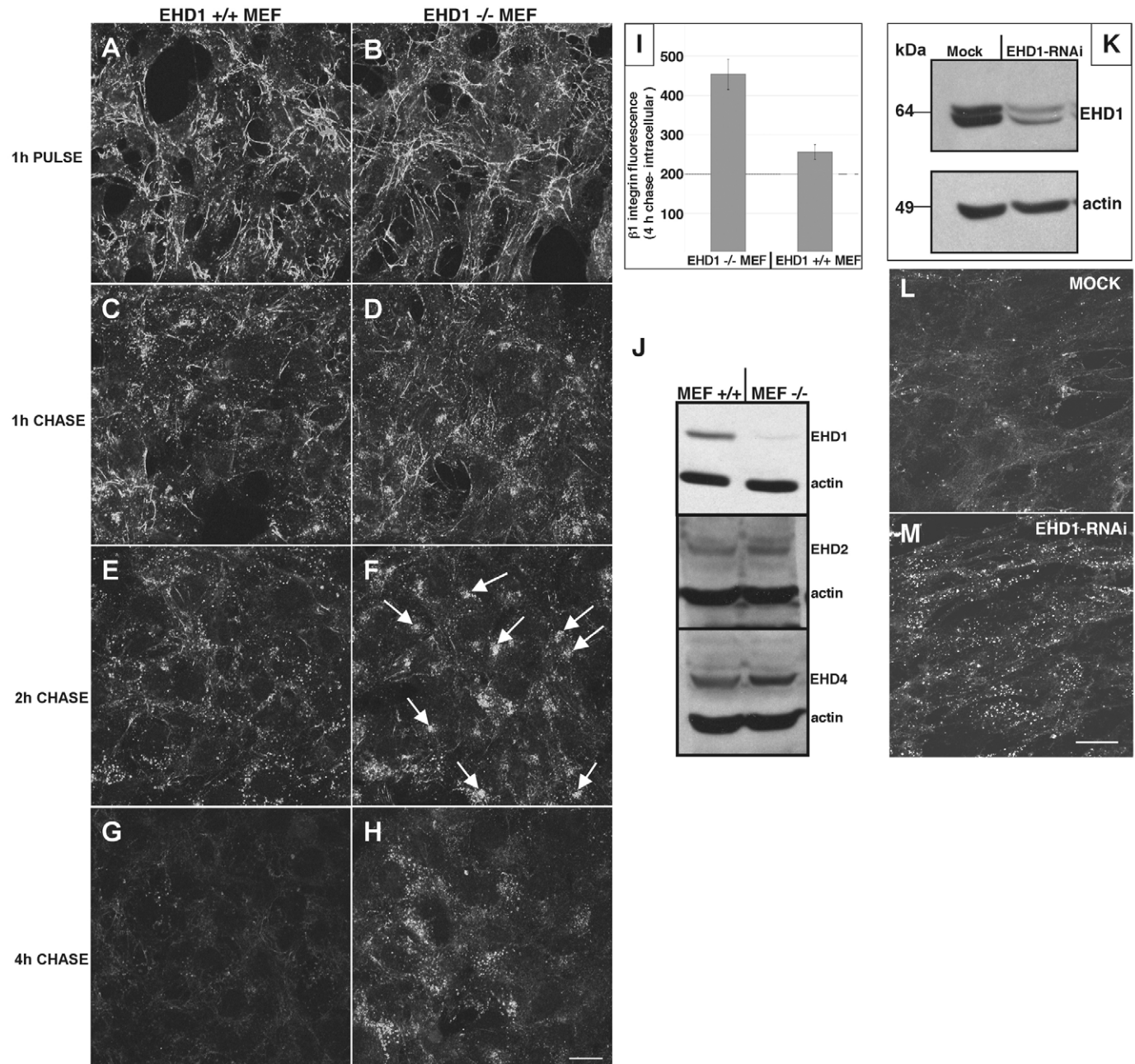
appears to be the primary cause for the impaired  $\beta 1$  integrin recycling in the fibroblasts.

We next assayed human fibroblast cells by treating them with mock- or EHD1-RNAi (Fig. 3K–M). As demonstrated by

immunoblotting, the efficacy of EHD1-knockdown was >90% (Fig. 3K). Although it is possible that the upper band of the doublet results from detection of EHD2 and/or EHD4 (which have a slightly higher molecular mass), our previous studies indicate that the EHD1 anti-peptide antibodies are specific for EHD1 (Naslavsky et al., 2006). Moreover, preliminary experiments suggest that this band is a phosphorylated form of EHD1 (M. Sharma and S.C., unpublished data). Having achieved significant reduction of endogenous EHD1 expression, we then subjected mock- and EHD1-RNAi-treated human fibroblasts to pulse-chase analysis with 12G10 anti- $\beta 1$  integrin antibodies (Fig. 3L,M). In mock-treated cells most of the  $\beta 1$  integrin appeared to have recycled back to the plasma membrane within 2 hours of chase, with some staining remaining in intracellular vesicles (Fig. 3L). By contrast, at this time point, EHD1-RNAi-treated cells displayed a considerably higher level of integrin-containing punctate endosomal structures (Fig. 3M). Collectively, these data suggest that loss of EHD1 results in delayed recycling of  $\beta 1$  integrins to the plasma membrane.

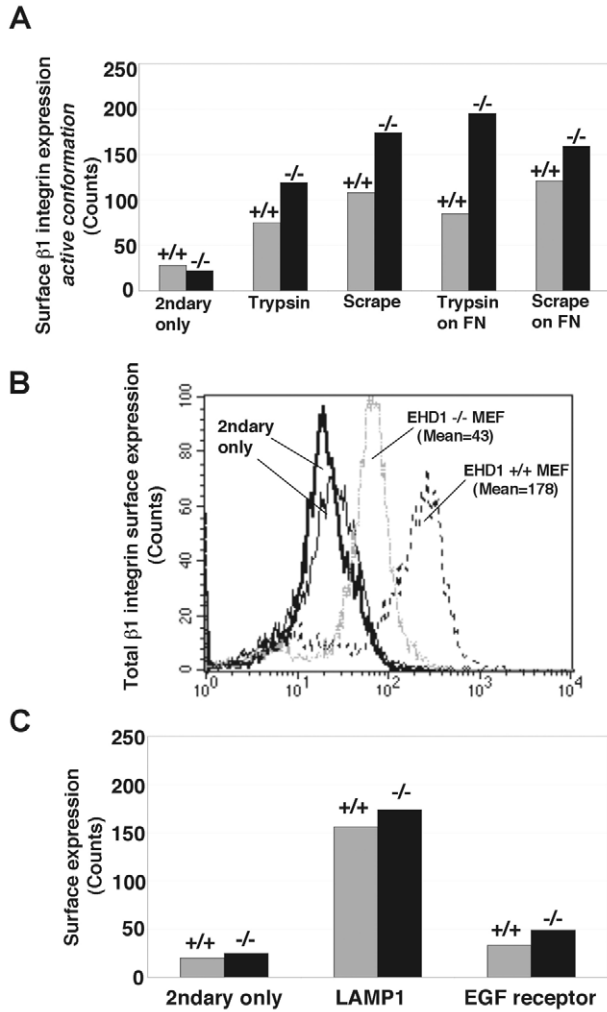
$\beta 1$  integrins play a crucial role in various cellular functions, including the regulation of focal adhesions, cell adherence to the extracellular matrix, cell spreading and cell motility (Hynes, 2002). To further appreciate the role of EHD1 and its impact upon  $\beta 1$  integrin trafficking and effects mediated downstream, we now sought to determine whether the cell-surface level of  $\beta 1$  integrin expression was altered in *Ehd1*<sup>−/−</sup> MEF cells compared with *Ehd1*<sup>+/+</sup> MEF cells. Accordingly, *Ehd1*<sup>+/+</sup> MEF and *Ehd1*<sup>−/−</sup> MEF cells growing either on plastic or FN were assayed for  $\beta 1$  integrins at the plasma membrane by flow cytometry experiments (Fig. 4A), using 9EG7 anti-mouse  $\beta 1$  integrin antibody that preferentially recognizes a ligand-bound conformational form of the integrin (Bazzoni et al., 1995). Since MEF cells are adherent, they were detached from non-coated or FN-coated plates by either brief trypsinization or cell scraping (Fig. 4A), or by brief incubation with EDTA (M.J. and S.C., unpublished data). In all cases, the level of activated  $\beta 1$  integrins at the plasma membrane was higher in the *Ehd1*<sup>−/−</sup> MEF cells, generally displaying an increase of 50–100%. However, when  $\beta 1$  integrin surface levels were assayed using a conformation-independent MB1.2 antibody, we observed that the overall  $\beta 1$  integrin levels were decreased on *Ehd1*<sup>−/−</sup> MEF cells (Fig. 4B). However, cell-surface levels of the lysosomal membrane protein LAMP1, which also localizes to the plasma membrane, were only slightly higher in *Ehd1*<sup>−/−</sup> MEF cells (Fig. 4C). Epidermal growth factor receptor (EGFR) also showed a modest increase in MEF *Ehd1*<sup>−/−</sup> cells at the plasma

membrane, indicating that it too may be regulated through the EHD1 endocytic pathway (Fig. 4C). Overall, these results indicate that the loss of EHD1 leads to decreased  $\beta 1$  integrin levels on the plasma membrane, probably because of



**Fig. 3.** Loss of EHD1 in MEF cells and human fibroblasts causes accumulation of  $\beta 1$  integrins in internal vesicles. MEF cells were pulsed with 9EG7 monoclonal antibodies that preferentially recognize ligand-bound mouse  $\beta 1$  integrins for 1 hour at 37°C (A–H). The cells were then briefly acid-stripped to remove non-internalized antibody, and then either fixed/permeabilized (1-hour pulse; A,B) or chased for 1, 2 or 4 hours (C–H). At the end of every chase, cells were stripped again to remove any recycled  $\beta 1$  integrins from the plasma membrane and the remaining intracellular  $\beta 1$  integrins were detected by immunostaining with Alexa Fluor 568 anti-rat secondary antibody. Arrows depict accumulation of  $\beta 1$  integrins at the ERC in *Ehd1*<sup>-/-</sup> MEFs (F). Levels of intracellular  $\beta 1$  integrins in *Ehd1*<sup>+/+</sup> MEF (G) and *Ehd1*<sup>-/-</sup> MEF cells (H) were quantified (graph in I) by the LSM 5 Pascale software using the Profile function. Representative fields (comprising more than 100 cells) were profiled by measuring the mean fluorescence in the field (~80  $\mu\text{m}$ ) every 4  $\mu\text{m}$ , and obtaining a mean value for all sections sampled. Standard deviations are depicted in the error bars, and the Student's *t*-test values for significance calculated at  $P < 0.0001$ . MEF cells were grown in culture, lysed, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot analysis (J). Human fibroblast cells growing on culture dishes (K) or coverslips (L,M) were treated with mock-RNAi or RNAi specific for EHD1 for 48 hours. Efficacy of RNAi was confirmed by immunoblotting with anti-EHD1 (K; top panel) and anti-actin as a control for equal protein loading (K; bottom panel). Mock-treated (L) and EHD1-RNAi-treated (M) human fibroblasts on coverslips were incubated with anti-human  $\beta 1$  integrin antibodies (MCA2028; Serotec) for 2 hours, briefly acid-stripped and chased with complete media for 2 hours. Intracellular  $\beta 1$  integrins were visualized by confocal microscopy following incubation with Alexa Fluor 568 anti-mouse secondary antibody under permeabilizing conditions. The micrographs shown are from a representative experiment from four independent experiments. Bars, 10  $\mu\text{m}$ .

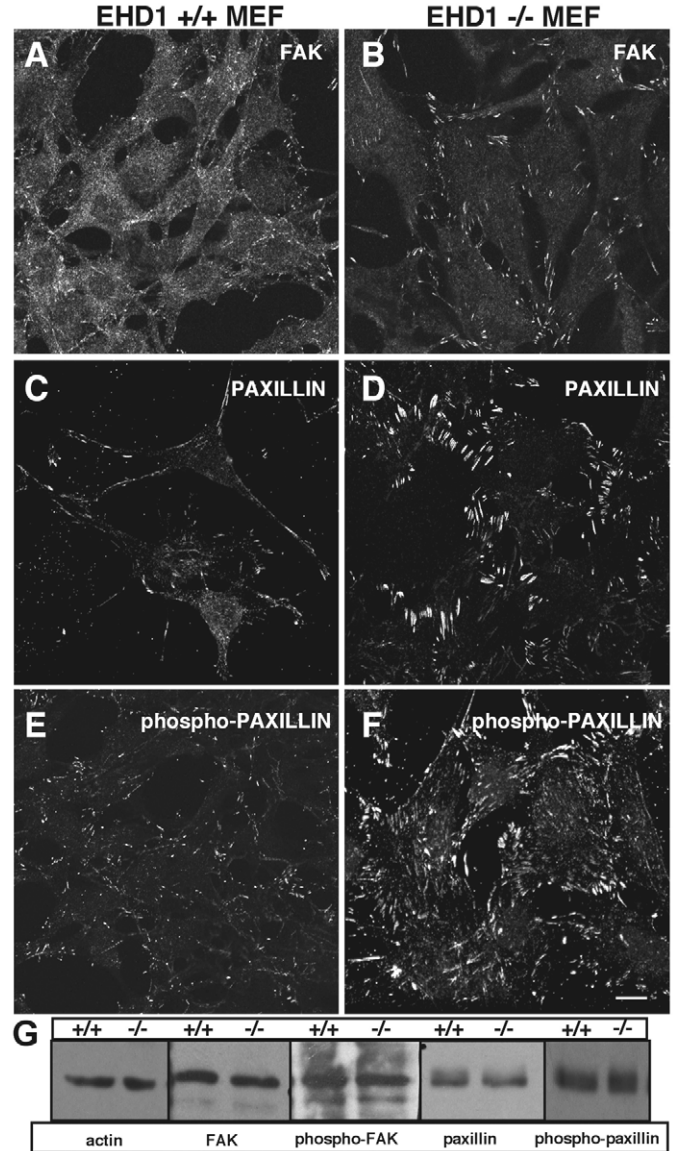




**Fig. 4.** Increased levels of activated  $\beta 1$  integrins but lower overall levels of  $\beta 1$  integrins are expressed on the plasma membrane of  $Ehd1^{-/-}$  MEF cells. MEF cells grown on uncoated (A–C) or fibronectin-coated (A; FN) plates were harvested by brief trypsinization (A; Trypsin and B,C) or scraping (A; Scrape), fixed and subjected to flow cytometry analysis under non-permeabilizing conditions with secondary antibody only (A–C) or first with 9EG7 antibodies that preferentially recognize ligand-bound  $\beta 1$  integrins (BD Pharmingen) (A), conformation-independent  $\beta 1$  integrin antibodies (MB1.2, Chemicon) (B), anti-LAMP1 antibodies (C) or antibodies directed against the epidermal growth factor (EGF) receptor (C) prior to secondary antibodies. The graphs depicted are representative of 4–6 independent experiments.  $+/+$ ,  $Ehd1^{+/+}$  MEF cells;  $-/-$ ,  $Ehd1^{-/-}$  MEF cells.

sequestering in an intracellular compartment. However, the increased localization of  $\beta 1$  integrins in an active conformation on the plasma membrane in  $Ehd1^{-/-}$  MEF cells raised the possibility of differences in focal adhesion dynamics in the absence of EHD1.

$\beta 1$  integrins play an important role in the regulation of focal adhesion complexes. To study the potential impact of EHD1 upon focal adhesions, we analyzed and compared the localization of several focal adhesion constituent proteins in  $Ehd1^{+/+}$  MEF and  $Ehd1^{-/-}$  MEF cells (Fig. 5). In  $Ehd1^{+/+}$  MEF



**Fig. 5.** The distribution of focal adhesion proteins in  $Ehd1^{-/-}$  MEF cells is altered at steady-state. MEF cells were immunostained with antibodies to FAK (A,B), paxillin (C,D) or phospho-paxillin (E,F) and analyzed by confocal microscopy. Lysates containing an equal amount of protein from  $Ehd1^{+/+}$  and  $Ehd1^{-/-}$  MEF cells were immunoblotted with antibodies against actin, FAK, phosphorylated FAK, paxillin or phosphorylated paxillin (G). Bar, 10  $\mu$ m.

cells at steady state, focal adhesion kinase (FAK) was observed both in the cytosol as well as at the plasma membrane in focal adhesion plaques (Fig. 5A). However,  $Ehd1^{-/-}$  MEF cells displayed less FAK in the cytosol, with the majority of the protein localized to distinct focal adhesions at the plasma membrane (Fig. 5B). Another well-characterized focal adhesion protein, paxillin, also showed significantly more prominent and longer focal adhesion structures (as well as less cytosolic localization) in the  $Ehd1^{-/-}$  MEF cells compared with their normal MEF counterparts (Fig. 5C,D). A similarly altered subcellular localization was also observed using antibodies

directed against the phosphorylated form of paxillin, also a key resident of focal adhesions (Brown et al., 2002) (Fig. 5E,F). Altered subcellular localization appeared to be the primary explanation for this phenomena, since neither enhanced expression nor increased phosphorylation of focal adhesion constituents was observed in *Ehd1*<sup>-/-</sup> MEF cells (Fig. 5G). Other focal adhesion resident proteins, including vinculin and the tyrosine 397 phosphorylated form of FAK (Mitra et al., 2005), were also associated with more prominent focal adhesions in cells lacking EHD1 (M.J. and S.C., unpublished data).

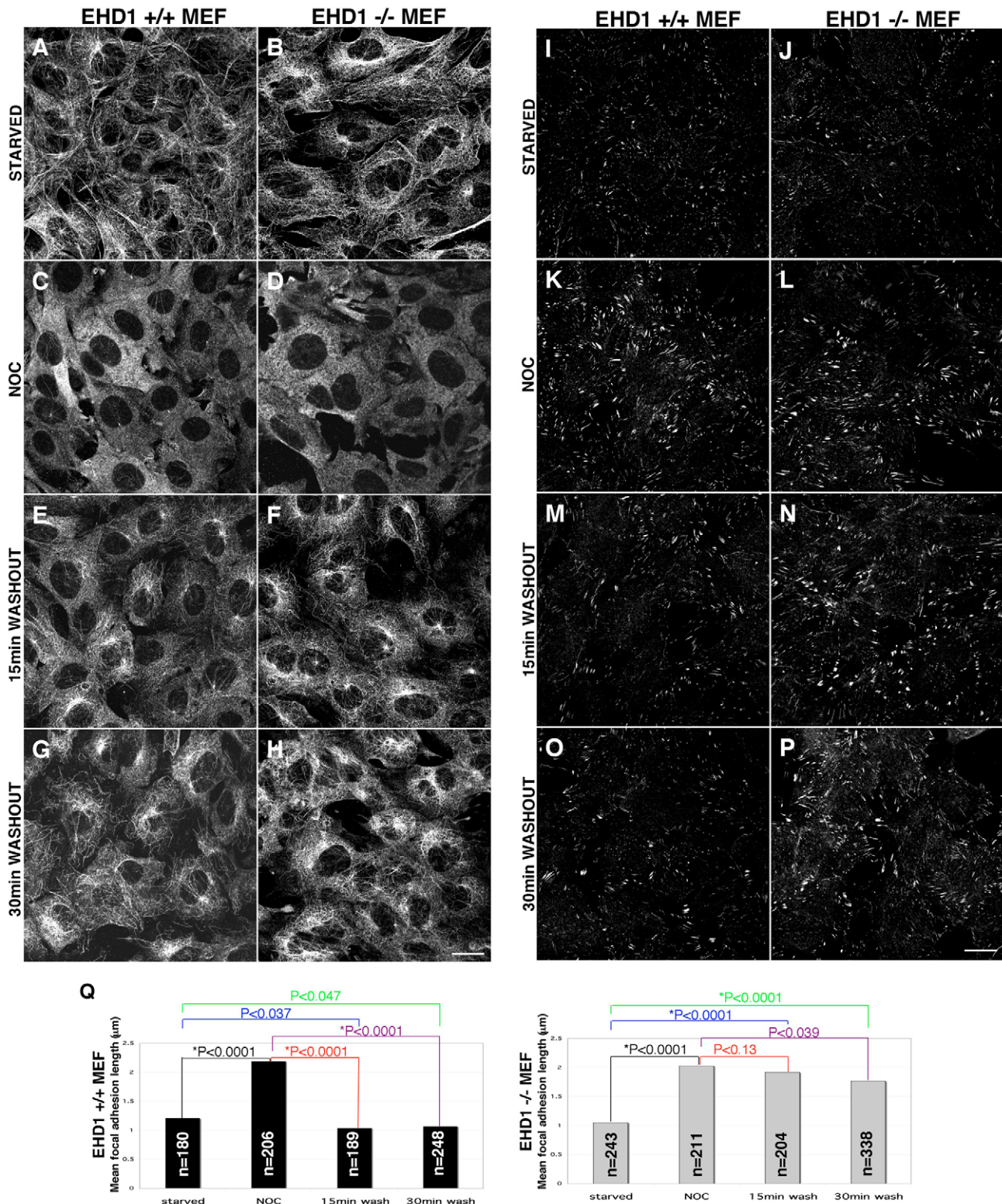
An explanation for the prominent focal adhesions in *Ehd1*<sup>-/-</sup> MEF cells might be a change in the kinetics of focal adhesion assembly and/or disassembly. To determine whether focal adhesion disassembly is impaired in *Ehd1*<sup>-/-</sup> MEF cells, we synchronized the formation of focal adhesions and monitored their disassembly by an immunofluorescence-based assay (Ezratty et al., 2005; Franco et al., 2004). *Ehd1*<sup>+/+</sup> MEF and *Ehd1*<sup>-/-</sup> MEF cells grown on FN-coated coverslips were incubated with nocodazole in the absence of serum, which induces the formation of focal adhesions through Rho-GTPase activity (Bershadsky et al., 1996; Liu et al., 1998) in addition to preventing microtubule polymerization. Serum-starved MEF cells displayed an elaborate microtubule network (Fig. 6A,B). Upon nocodazole treatment, the microtubules were disrupted and the tubulin distribution pattern was largely cytosolic for both *Ehd1*<sup>+/+</sup> MEF and *Ehd1*<sup>-/-</sup> MEF cells (Fig. 6C,D). As the effect of nocodazole is reversible, we washed-out the drug and allowed microtubule polymerization to recover for 15–30 minutes (Fig. 6E–H). Having shown the efficacy of nocodazole and its reversible nature, we now turned to study the formation and disassembly of focal adhesions over time in this system, using paxillin as a representative component of focal adhesions. Serum-starved *Ehd1*<sup>+/+</sup> and *Ehd1*<sup>-/-</sup> cells displayed weak focal adhesion staining and a mean focal adhesion length of ~1 μm each (Fig. 6I,J,Q). The intensity of focal adhesion staining was greatly enhanced for both cell lines upon addition of nocodazole, with the mean focal adhesion length having doubled to approximately 2 μm (Fig. 6K,L,Q). Upon 15–30 minutes of nocodazole washout, *Ehd1*<sup>+/+</sup> MEF cells displayed a sharp reduction in both focal adhesion intensity and mean length because of their disassembly (Fig. 6M,O), approximating the focal adhesion intensity observed in starved, pre-nocodazole-treated cells (compare with Fig. 6I) and again displaying a mean focal adhesion length of ~1 μm (Fig. 6Q). By contrast, the reduction in focal adhesion intensity and mean length following washout was markedly delayed in *Ehd1*<sup>-/-</sup> MEF cells, with little change even after 30 minutes of washout (Fig. 6N,P,Q). These data suggest that focal adhesion disassembly is impaired in cells lacking EHD1 expression.

Integrin trafficking is crucial for the execution of integrin-mediated processes, such as adherence, cell spreading and motility (Caswell and Norman, 2006; Jones et al., 2006; Pellinen and Ivaska, 2006). Given that loss of EHD1 slows the dynamics of focal adhesion turnover in *Ehd1*<sup>-/-</sup> MEF cells, we sought to determine whether integrin-mediated motility is influenced by the loss of EHD1. We compared the migration of *Ehd1*<sup>+/+</sup> and *Ehd1*<sup>-/-</sup> MEF cells on FN- and VN-coated coverslips over 12 hours on a 'scratch-assay' (Fig. 7). On FN, which engages β1 integrins, the *Ehd1*<sup>+/+</sup> MEF cells migrated

into the scratched area, closing most of the wound and colonizing 65% of the wound area (Fig. 7C). At the same time, the *Ehd1*<sup>-/-</sup> MEF cells colonized only 15% of the scratched region (Fig. 7D). When plated on VN, a ligand for αvβ3 integrins, both *Ehd1*<sup>+/+</sup> (Fig. 7E) and *Ehd1*<sup>-/-</sup> MEF cells (Fig. 7F) showed similar degrees of colonization (26% and 22%, respectively). This level of migration on VN was significantly less than the 65% colonization achieved by *Ehd1*<sup>+/+</sup> MEF cells on FN, highlighting the role of EHD1 in regulating β1 integrin trafficking and downstream function. A previous study (Rapaport et al., 2006) and our current experiments (our unpublished observations) show the proliferation rates of *Ehd1*<sup>+/+</sup> MEF and *Ehd1*<sup>-/-</sup> MEF to be very similar, rendering the enhanced wound colonization by *Ehd1*<sup>+/+</sup> MEF cells unlikely to be because of differences in cell growth within 12 hours. RNAi-knockdown of EHD1 in *Ehd1*<sup>+/+</sup> MEF cells also delayed migration, although the difference was not as marked (M.J. and S.C., unpublished data). To determine whether EHD1-knockdown alters the localization of β1 integrins on the cell surface of migrating cells, we compared the distribution of β1 integrins at the leading edge of *Ehd1*<sup>+/+</sup> and *Ehd1*<sup>-/-</sup> MEF cells plated on FN (Fig. 7G,H). As shown, the *Ehd1*<sup>+/+</sup> cells displayed a large number of β1 integrin-containing filopodial spikes that projected from the plasma membrane towards the wound area (Fig. 7G and inset). By contrast, *Ehd1*<sup>-/-</sup> MEF cells exhibited few filopodia extending towards the wound, but displayed levels of integrins at the plasma membrane as well as being particularly pronounced in intracellular structures (Fig. 7H and inset). Overall, these findings define a role for EHD1 in regulating integrin-mediated cell motility, and suggest that the function of β1 integrins is more tightly regulated by EHD1-knockdown than that of β3 integrins.

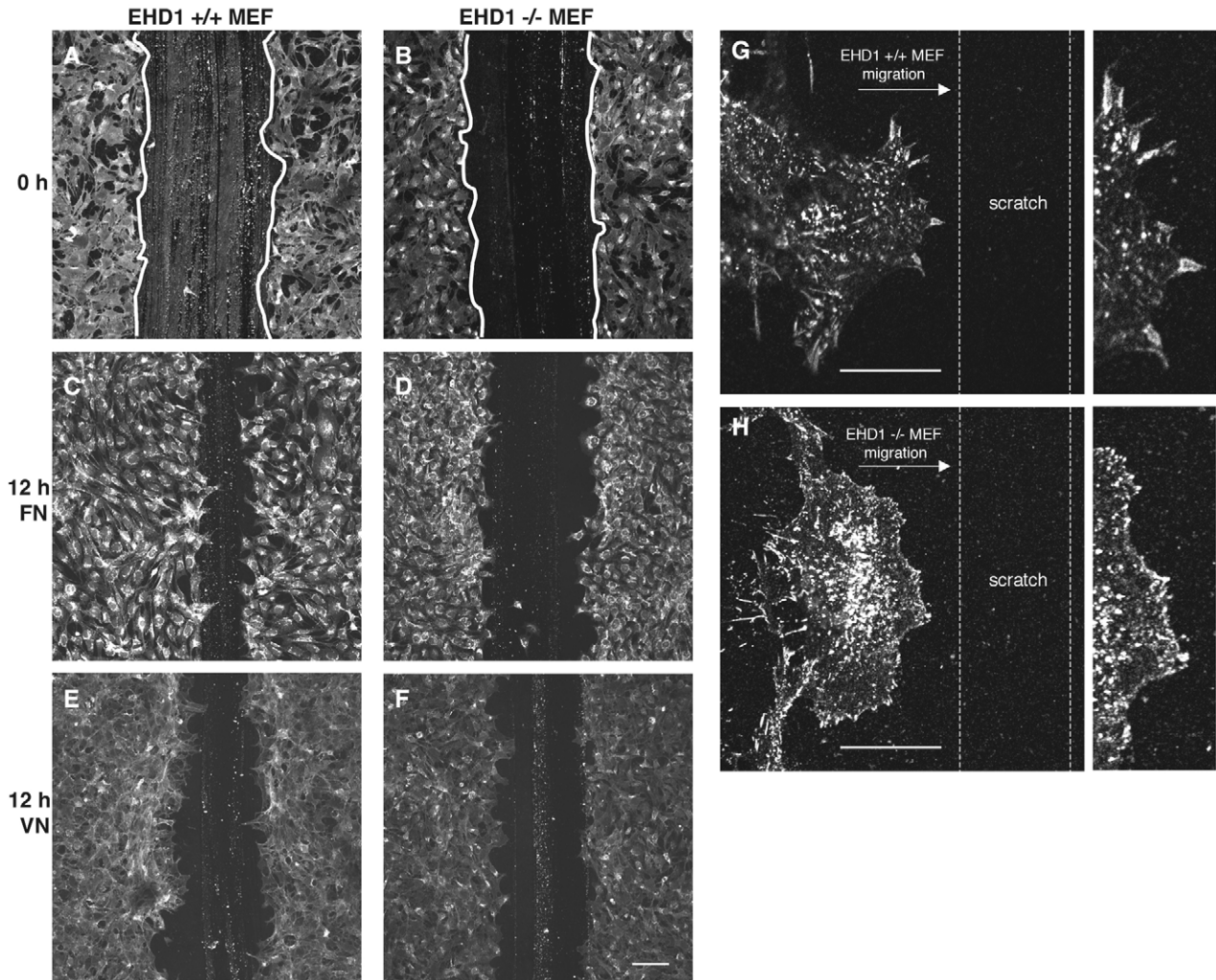
To further examine the functional significance of EHD1-mediated control of β1 integrin trafficking, we compared the ability of *Ehd1*<sup>+/+</sup> MEF and *Ehd1*<sup>-/-</sup> MEF cells to spread on FN (Fig. 8). We calculated that 80 randomly chosen *Ehd1*<sup>+/+</sup> MEF cells yielded a mean surface area of ~2300 μm<sup>2</sup> after 3 hours of spreading (Fig. 8A,E). By contrast, the same number of *Ehd1*<sup>-/-</sup> MEF cells spread less at this time point, and their mean surface area was calculated at only approximately 1500 μm<sup>2</sup> (Fig. 8B,E). Differences of up to threefold in spreading were observed in individual experiments, depending on the time the cells were allowed to spread prior to fixation. Upon introduction of a GFP-EHD1 construct into *Ehd1*<sup>-/-</sup> MEF cells, the rate of cell spreading was partially rescued (Fig. 8C,E; green denotes transfected cells) when compared with neighboring non-transfected cells (red-only cells). Lack of complete rescue probably results from heterogeneous expression levels of the transfected GFP-EHD1 in the *Ehd1*<sup>-/-</sup> MEF cells. As an additional control, we used a GFP-EHD1 mutant with a point mutation in a conserved glycine residue (EHD1 G65R) crucial for nucleotide binding (Lee et al., 2005; Naslavsky et al., 2006) and localization of EHD1 to membranes, rendering the mutant EHD1 dysfunctional (Caplan et al., 2002; Grant et al., 2001; Lin et al., 2001). As shown, GFP-EHD1 G65R was incapable of rescuing cell spreading in *Ehd1*<sup>-/-</sup> MEF cells (Fig. 8D,E), and even showed a modest additional decrease in cell spreading. Collectively, these data support a role for EHD1 in regulating β1 integrin trafficking and downstream functions, such as cell spreading and migration.





**Fig. 6.** Slower disassembly of focal adhesions in *Ehd1*<sup>-/-</sup> MEF cells. MEF cells were plated on fibronectin-coated coverslips, serum-starved and fixed (A,B; I,J) or treated with 10  $\mu$ M nocodazole (NOC) for 15 minutes at 37°C (C-H; K-P). For some coverslips, NOC was washed out by incubating the cells in starvation media in the absence of the drug for an additional 15 minutes (E,F; M,N) or 30 minutes (G,H; O,P). After fixation, cells were immunostained with either mouse anti-tubulin (A-H) or mouse anti-paxillin (I-P). (Q) Quantification of focal-adhesion length in *Ehd1*<sup>-/-</sup> and *Ehd1*<sup>+/+</sup> cells was done by using Zeiss LSM 5 Pascale software. The number of focal adhesions measured is indicated, and Student's *t*-tests were applied to determine the significance of differences in mean focal adhesion lengths. Differences exhibiting a high degree of significance ( $P < 0.0001$ ) are noted with asterisks. This is a representative experiment from four independent experiments. Bars, 10  $\mu$ m.



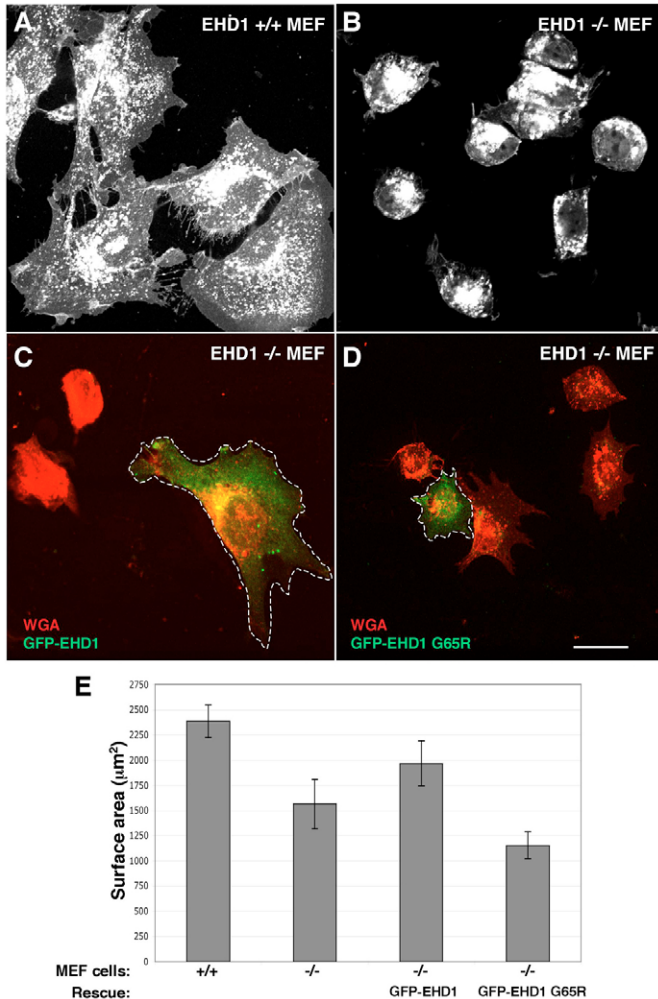


**Fig. 7.** Loss of EHD1 expression results in impaired cell motility. Equal numbers of *Ehd1*<sup>+/+</sup> MEF (A,C,E) and *Ehd1*<sup>-/-</sup> MEF cells (B,D,F), were plated on fibronectin (FN)-coated coverslips (C,D) or vitronectin (VN)-coated coverslips (E,F). Scratches of ~700 μm were introduced on the coverslips, and the cells were either fixed immediately (A,B) or after an additional 12-hour incubation period (C-F). Cells were visualized with fluorochrome-labeled wheat germ agglutinin and confocal microscopy. The micrographs depicted are representative ones from six independent experiments. *Ehd1*<sup>+/+</sup> and *Ehd1*<sup>-/-</sup> MEF cells were plated on fibronectin-coated coverslips at sub-confluent concentrations (G,H) and scratched as in A-F. Eight hours after the scratch, cells were then pulsed with 9EG7 anti-β1 integrin antibody for an additional hour to visualize β1 integrins in the migrating cells. Arrows indicate the direction of the migration, and the scratch region is indicated by broken lines. This experiment is a representative one of five independent experiments. Bars, 200 μm (A-F); 10 μm (G,H).

## Discussion

Despite the crucial functions that integrins play in modulating the interaction of the cell with the extracellular matrix, until recently relatively little emphasis has been placed on understanding the subcellular itinerary of these proteins and determining the mode by which this itinerary is regulated. Given the importance of β1 integrin trafficking for a wide variety of cell functions, especially motility and migration, tumor cell invasiveness (reviewed by Caswell and Norman, 2006) and cell adhesion and spreading (Dunphy et al., 2006; Roberts et al., 2001), renewed focus is being placed on the identification of proteins involved in the regulation of integrin trafficking. We have demonstrated that the C-terminal EHD protein EHD1 plays an important role in modulating β1 integrin function by regulating recycling of these receptors.

Initially, using HeLa cells as a well-established model to study alterations in the subcellular itinerary of β1 integrins (Powelka et al., 2004), we demonstrated that RNAi-knockdown of EHD1 led to an accumulation of β1 integrins in a transferrin-containing perinuclear region corresponding to the ERC. Moreover, we detected membrane vesicles containing both internalized β1 integrins and EHD1. Accordingly, EHD1, whose primary role appears to be in regulating the return of endocytosed cargo to the plasma membrane from a Rab11-positive recycling compartment (Caplan et al., 2002; Grant et al., 2001; Lin et al., 2001; Naslavsky et al., 2004; Naslavsky et al., 2006; Rapaport et al., 2006), may play a similar and/or overlapping role to that of Rab11 in regulating the long loop of β1 integrin recycling (Roberts et al., 2001). Accumulation of intracellular β1 integrins was also documented upon RNAi-



**Fig. 8.** Cell spreading on fibronectin is impaired in the absence of EHD1. *Ehd1*<sup>+/+</sup> MEF (A), *Ehd1*<sup>-/-</sup> MEF (B), *Ehd1*<sup>-/-</sup> MEF cells transfected with GFP-EHD1 (C) or *Ehd1*<sup>-/-</sup> MEF cells transfected with GFP-EHD1 G65R (D) were plated on coverslips coated with 10  $\mu\text{g}/\text{ml}$  fibronectin and allowed to spread for 3 hours in complete media. Cells were then fixed and stained with Cy3 wheat germ agglutinin (WGA; red) (A–D). Cell surface boundaries were outlined for 80–100 individual cells chosen randomly [*Ehd1*<sup>+/+</sup> MEF cells, *Ehd1*<sup>-/-</sup> MEF cells and *Ehd1*<sup>-/-</sup> cells transfected with GFP-EHD1 (green)], and 40 cells for *Ehd1*<sup>-/-</sup> MEF cells transfected with GFP-EHD1 G65R (green). LSM 5 Pascal software was used to calculate the mean surface area of each population (see error bars for standard deviation) (E). One-tailed Student's *t*-tests were applied to test statistical significance of the data, with the *P* values for *Ehd1*<sup>+/+</sup> versus *Ehd1*<sup>-/-</sup> MEF being *P* < 0.001; *Ehd1*<sup>+/+</sup> versus *Ehd1*<sup>-/-</sup> MEF transfected with GFP-EHD1 G65R being *P* < 0.0001; *Ehd1*<sup>-/-</sup> versus *Ehd1*<sup>-/-</sup> MEF transfected with GFP-EHD1 being *P* < 0.0001; and *Ehd1*<sup>-/-</sup> MEF transfected with GFP-EHD1 versus *Ehd1*<sup>-/-</sup> MEF transfected with GFP-EHD1 G65R being *P* < 0.0001. This is a representative experiment from four independent experiments showing a similar trend. Bar, 10  $\mu\text{m}$ .

knockdown of the EHD1 binding partner, Rabenosyn-5 (M.J. and S.C., unpublished data), similar to the accumulation observed for internalized transferrin (Naslavsky et al., 2004). Since it has recently been demonstrated that Rab21 and Rab5

associate with integrins (Pellinen et al., 2006), and Rabenosyn-5 is a divalent effector of both Rab4 and Rab5 (Nielsen et al., 2000) (and possibly also Rab21), one might speculate that integrin endocytosis may be coordinated jointly by GTP-binding proteins such as Rab21/Rab5 and the C-terminal EHD1 protein through interactions with common effectors such as Rabenosyn-5. Although the precise mechanism of Rabenosyn-5 function is not clear, it has been implicated in vesicle fusion (Nielsen et al., 2000). Interestingly, integrins such as  $\alpha 5\beta 1$  also accumulate in a Rab11-containing compartment when SNARE activity is inhibited (Skalski and Coppolino, 2005), reinforcing the notion that SNARE-mediated fusion is important in the regulation of integrin recycling.

Although HeLa cells served as an established model for the initial assessment of EHD1 in  $\beta 1$  integrin trafficking (Becker and Hannun, 2003; Powelka et al., 2004), integrin function has been studied more extensively in fibroblasts. Using MEF cells derived from mice lacking EHD1 expression (Rapaport et al., 2006), we examined the role of EHD1 in regulating the trafficking of  $\beta 1$  integrins. Loss of EHD1 led to an accumulation of intracellular  $\beta 1$  integrins observed after 2 hours of chase, when compared with  $\beta 1$  integrins in normal *Ehd1*<sup>+/+</sup> MEF counterpart cells. Moreover, after 4 hours of chase, an approximately twofold increase was observed in the level of non-recycled  $\beta 1$  integrins. The role of EHD1 in regulating  $\beta 1$  integrin recycling was further supported by flow cytometry experiments with the MB1.2 antibody (which recognizes conformation-independent  $\beta 1$  integrins), demonstrating that overall levels of  $\beta 1$  integrins are reduced on the cell surface as a result of impaired recycling (Fig. 4B). To further validate the role of EHD1 in  $\beta 1$  integrin recycling, an RNAi approach was applied to a human fibroblast cell line and yielded similar results. Morphologically, the accumulation of  $\beta 1$  integrins at the ERC in the human fibroblasts had a more dispersed pattern than in HeLa and MEF cells. Indeed, the ERC in the human fibroblasts is not as well defined and not relegated exclusively to the perinuclear region as observed in HeLa, CHO and some other cell types. These data provide evidence promoting a role for EHD1 in the regulation of  $\beta 1$  integrin trafficking.

To transduce signals into the cell, integrins localized to the plasma membrane first need to recruit a series of intracellular proteins and assemble them into small plaques known as focal complexes or focal adhesions (Wozniak et al., 2004). Recent studies have shown that focal adhesion formation is a complicated process, entailing the sequential recruitment of multiple proteins with different kinetics (Webb et al., 2003). Focal adhesion disassembly involves microtubules, dynamin and FAK (Ezratty et al., 2005), and the protease calpain (Franco et al., 2004). Our data demonstrate that *Ehd1*<sup>-/-</sup> MEF cells exhibit more prominent focal adhesions [determined by staining for focal adhesion constituent proteins such as paxillin (Brown et al., 2002; Schaller, 2001; Turner, 2000) and FAK (Mittra et al., 2005)], resulting from slower disassembly kinetics. Interestingly, similar to the loss of EHD1 expression, interference with the function of the small GTPase Rab21 (by use of a dominant-negative GDP-locked mutant) also induced prominent focal adhesions (Pellinen et al., 2006). Although it is not understood how the absence of EHD1 stabilizes focal adhesions, our findings that there is no enhancement in the



levels of phosphorylated paxillin and phosphorylated FAK in *Ehd1*<sup>-/-</sup> MEF cells, along with preliminary data suggesting that calpain activity is not affected in *Ehd1*<sup>-/-</sup> MEF cells (M.J. and S.C., unpublished data), support the notion that impaired recycling of  $\beta$ 1 integrins or one of the many factors involved in focal adhesion disassembly may be responsible for these phenomena. Moreover, the increased levels of active  $\beta$ 1 integrins detected at the plasma membrane of the *Ehd1*<sup>-/-</sup> MEF cells (Fig. 4A) may also be related to the delay in focal adhesion disassembly.

The recycling of integrins as well as assembly and disassembly of focal adhesions are crucial for polarized cell motility (Webb et al., 2004) (reviewed by Wozniak et al., 2004). Cell motility depends on the internalization of integrins at the rear of a migrating cell and their subsequent recycling to the plasma membrane at the leading edge of the cell (Bretscher, 1992; Lawson and Maxfield, 1995; Strachan and Condic, 2004). It has been further suggested that this activity involves elaborate interactions between Arf6 and ArfGAP complexes that are proposed to recruit paxillin and other focal adhesion constituents to newly assembling focal adhesions containing Rac-induced microfilaments at the leading edge of the cell (reviewed by de Curtis, 2001). Indeed, a recent report demonstrates that RNAi-knockdown of ACAP1, a GTPase-activating protein (GAP) for Arf6 (Jackson et al., 2000), inhibits  $\beta$ 1 integrin recycling and impairs cell migration on a FN-coated matrix (Li et al., 2005). In accordance with these findings, another study shows that loss of expression of the Arf6 GTP exchange factor (GEF), GEP100/BRAG2, causes enhanced cell attachment and spreading on FN-coated substrates (Dunphy et al., 2006). Further evidence of Arf6 involvement in the regulation of integrin trafficking is supported by the recent finding that recycled  $\beta$ 1 integrins colocalize with Arf6 in peripheral membrane ruffles (Powelka et al., 2004). We have previously shown a substantial colocalization between Arf6 and EHD1, and that the subcellular distribution of EHD1 is dramatically altered in response to the nucleotide status of Arf6 and to the expression of both ACAP1 and EFA6 (Caplan et al., 2002), Arf6 GAPs and GEFs, respectively. It is noteworthy that both the stabilization of focal adhesions and the significant decrease observed in the rate of fibroblast migration in fibroblasts lacking EHD1 in this study are similar to the effects caused by transfection of a dominant-negative Rab21 (Pellinen et al., 2006). In addition, the combination of loss of polarized filopodia and delayed migration that we observed in *Ehd1*<sup>-/-</sup> MEF cells when grown on FN is reminiscent of the effects documented for FAK<sup>-/-</sup> MEFs (Ilic et al., 1995).

Although it is presently unclear why delayed  $\beta$ 1 integrin recycling stabilizes focal adhesions, the most likely possibility is that the trafficking of some of the proteins playing key roles in focal adhesion disassembly is regulated through an EHD1-mediated recycling pathway. Potential examples include FAK, Src, p130CAS, extracellular signal-related kinase (ERK) and myosin light chain kinase (Webb et al., 2004) or Endo180 (Sturge et al., 2006). Fibroblasts lacking EHD1 exhibit a considerable delay in their spreading on a FN-coated surface, and this phenotype can be rescued by the introduction of wild-type EHD1, implying that this loss of protein function is also a primary cause of defective cell spreading.

Our data point to a role for EHD1 in the control of  $\beta$ 1

integrin trafficking, adding this endocytic protein to the growing list of proteins that regulate the subcellular itinerary of  $\beta$ 1 integrins. The precise mechanisms of the complex physical and functional interactions between EHD1, Rab4, Rab11, Arf6 and their effectors remain to be elucidated, and a better understanding of their coordinated control of endocytic transport will shed new light on the trafficking and function of integrins.

## Materials and Methods

### Antibodies and reagents

All cDNA constructs have been described previously (Caplan et al., 2002; Naslavsky et al., 2004). The following antibodies were used: 12G10 mouse monoclonal antibodies to human  $\beta$ 1 integrins, with higher affinity to the activated conformation (Mould et al., 1995; Powelka et al., 2004) (cat. # MCA2028, Serotec, Oxford, UK), LAMP1 (Santa Cruz Biotechnology, Santa Cruz, CA),  $\beta$ -actin (Novus Biologicals, Littleton, CO),  $\alpha$ -tubulin (Invitrogen, Carlsbad, CA), FAK (Upstate, Lake Placid, NY), paxillin and EGFR (BD Transduction Laboratories, Franklin Lakes, NJ), rabbit phospho-specific antibodies to paxillin phosphorylated at Y118 and FAK phosphorylated at Y397 (Biosource, Camarillo, CA), rat 9EG7 monoclonal antibodies that preferentially recognize ligand-bound mouse  $\beta$ 1 integrins (Bazzoni et al., 1995) (cat. # 550531, BD Pharmingen, San Diego, CA), and conformation-independent MB1.2 rat monoclonal antibodies against mouse  $\beta$ 1 integrins (Sakai et al., 1998; Von Ballestrem et al., 1996) (cat. # MAB1997, Chemicon, Temecula, CA). Affinity-purified rabbit polyclonal antibodies against human EHD1 (DLPPHLVPPSKRRHE), EHD2 (VERGPDEAMEDGEEGSD) and EHD4 (SHRKSLPKAD) (AnaSpec, San Jose, CA) were prepared. Cy-3-conjugated anti-mouse and anti-rabbit immunoglobulin G (IgG), Alexa Fluor 488-conjugated antibody to mouse and rabbit IgG, Alexa Fluor 633 goat anti-mouse, Alexa Fluor 633 goat anti-rat, Alexa Fluor 568 goat anti-rat, transferrin-Alexa-Fluor-568 and Cy-3-labeled wheat germ agglutinin (WGA) were from Invitrogen. Goat anti-mouse horseradish peroxidase (HRP) and donkey anti-rabbit HRP were obtained from Jackson ImmunoResearch (West Grove, PA).

### Cell culture

*Ehd1*<sup>-/-</sup> MEF cells were previously described (Rapaport et al., 2006). Human foreskin-derived fibroblasts were obtained from ATCC (BJ; CRL-2522, Manassas, VA). MEF and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (high glucose), whereas human foreskin fibroblasts were maintained in Eagle's minimal essential medium (EMEM). All of the media was supplemented with 10% fetal bovine serum (FBS), 100  $\mu$ g/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine.

### Gene knockdown by RNAi and transient transfection

Oligonucleotide duplexes targeting human EHD1 (bp 942–963: AAGAAA-GAGATGCCCAATGTC, Dharmacon, Lafayette, CO) or mouse EHD1 (bp 921–942: AAGACATCCAGTCTCTGCCGA, Dharmacon) were transfected into HeLa cells or *Ehd1*<sup>+/+</sup> MEF cells for 48 hours using Oligofectamine (Invitrogen, Carlsbad, CA) (Elbashir et al., 2001). SMARTpool oligonucleotide duplexes (Dharmacon), targeting human-EHD1, were transfected into human foreskin fibroblasts using Dharmafect (Dharmacon) for 48 hours. Efficacy of EHD1-RNAi was confirmed by immunoblotting lysates with antibodies for EHD1.

### $\beta$ 1 integrin assays and transferrin-recycling assays

HeLa and human fibroblast cells were grown on coverslips, and transfected with appropriate EHD1-RNAi oligonucleotides for 48 hours. Based on a modified protocol of Hsu and co-workers (Powelka et al., 2004), cells were starved for 2 hours at 37°C in DMEM lacking serum [but containing 0.5% bovine serum albumin (BSA)], followed by a 2-hour pulse with 5  $\mu$ g/ml antibody to human  $\beta$ 1 integrin at 37°C. Surface antibodies were removed by an acid rinse (0.5% acetic acid, 0.5 M NaCl, pH 3.0) for 45 seconds. The chase was performed in complete media (containing 10% FBS) at 37°C for 2 hours and cells were fixed with 4% (v/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS). *Ehd1*<sup>+/+</sup> MEF cells and *Ehd1*<sup>-/-</sup> MEF cells were processed in similar fashion, using a 1-hour pulse and 1–4 hours of chase.

For combined  $\beta$ 1 integrin/transferrin recycling assays, at the end of the 2-hour pulse with  $\beta$ 1 integrin antibodies, the cells were then pulsed for 10 minutes with transferrin-Alexa-Fluor-568, and chased for 15 minutes longer in complete media at 37°C prior to fixation. Fixed cells were then incubated with the appropriate fluorochrome-conjugated secondary antibody mixture containing 0.1% (w/v) saponin and 0.1% (w/v) BSA for 30 minutes. All images were acquired on an LSM 5 Pascal confocal microscope (Carl Zeiss, Thornwood, NY) by using a 63 $\times$ 1.4 numerical aperture objective with appropriate filters. For combined internalization studies with both internalized transferrin and  $\beta$ 1 integrins, 4  $\mu$ m z-sections were obtained and colocalizations were validated in three-dimensions.

To quantify  $\beta 1$  integrin recycling by flow cytometry, HeLa cells at sub-confluent density were treated with EHD1-RNAi or mock-treated. After 48 hours the cells were serum-starved for 2 hours, pulsed with anti- $\beta 1$  integrin antibody for 2 hours, acid-stripped and chased in complete media for 2 hours. Following trypsinization and pelleting, cells were incubated under non-permeabilizing conditions with Alexa Fluor 633-conjugated goat anti-mouse secondary antibody for 30 minutes, and fixed in 4% PFA/PBS. At least 10,000 cells were analyzed for reappearance of  $\beta 1$  integrins on the plasma membrane by flow cytometry analysis (BD Biosciences, San Jose, CA).

### Focal adhesion disassembly

MEF cells were serum-starved in DMEM with 0.5% BSA on glass coverslips for 72 hours prior to addition of 10  $\mu$ M nocodazole for 15 minutes at 37°C, and either fixed directly or first incubated in starvation media (Ezratty et al., 2005). Cells were then immunostained with either mouse monoclonal antibodies against paxillin or tubulin and appropriate secondary antibodies. Quantification of mean focal adhesion length was done using LSM 5 Pascal software for an average of 227 adhesions per treatment. One-tailed Student's *t*-tests were applied to test statistical significance of the data.

### Migration assay

MEF cells were grown at equivalent confluence for 18 hours on coverslips precoated with 10  $\mu$ g/ml FN or 16.7  $\mu$ g/ml VN. A single scratch of ~700  $\mu$ m was introduced with a pipette tip, and cells were allowed to migrate into the wound for 12 hours at 37°C. Cells were visualized by immunostaining with fluorochrome-labeled WGA and confocal microscopy using a 10 $\times$ 0.3 numerical aperture objective.

### Cell spreading

MEF cells were trypsinized and replated on coverslips precoated with 10  $\mu$ g/ml FN, and allowed to spread for 3 hours at 37°C in the presence of complete media. Cells were fixed and labeled with Cy-3-conjugated WGA for 30 minutes to better visualize the cell outlines. Cell surface boundaries were outlined for 80 individual cells chosen randomly and LSM 5 Pascal software was used to calculate the mean surface area and standard deviation of each population. One-tailed Student's *t*-tests were applied to test the statistical significance of the data.

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