Contact-dependent inhibition of EGFR signaling by Nf2/Merlin

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The neurofibromatosis type 2 (NF2) tumor suppressor, Merlin, is a membrane/cytoskeleton-associated protein that mediates contact-dependent inhibition of proliferation. Here we show that upon cell–cell contact Merlin coordinates the processes of adherens junction stabilization and negative regulation of epidermal growth factor receptor (EGFR) signaling by restraining the EGFR into a membrane compartment from which it can neither signal nor be internalized. In confluent *Nf2*$^{-/-}$ cells, EGFR activation persists, driving continued proliferation that is halted by specific EGFR inhibitors. These studies define a new mechanism of tumor suppression, provide mechanistic insight into the poorly understood phenomenon of contact-dependent inhibition of proliferation, and suggest a therapeutic strategy for *NF2*-mutant tumors.

Introduction

The identification and study of tumor suppressor genes has provided insight into the normal mechanisms of cell proliferation control (Sherr, 2004). Most tumor suppressors function intracellularly to control the cell division cycle; however, the interface between a cell and its environment also plays a critical role in tumor development and metastasis. The product of the neurofibromatosis type 2 (NF2) tumor suppressor gene, Merlin, localizes to and appears to act at this interface (McClatchey and Giovannini, 2005). Loss of NF2 function is associated with the development of multiple cancers in humans and mice (McClatchey et al., 1998; Giovannini et al., 2000; Baser et al., 2002). In humans, *NF2* mutations are associated with familial and sporadic nervous system tumors and with other sporadic cancers such as mesothelioma, whereas heterozygous *Nf2* mutant mice develop bone, liver, and other tumors that are highly metastatic. Treatment strategies for NF2 are currently limited given the often intractable location and multiplicity of tumors, together with their tendency to recur. Surgical approaches are the current standard therapy and pharmacological treatments are not available.

Merlin is closely related to the ERM (Ezrin/Radixin/Moesin) proteins that are thought to organize cortical membrane domains that interface with the extracellular environment, via linking membrane-associated proteins to the actin cytoskeleton (Bretscher et al., 2002; Lallemand et al., 2003; McClatchey, 2003; Ramesh, 2004). Although Merlin can functionally and physically interact with several proteins, including p21-activated kinase (Kissil et al., 2003; Hirokawa et al., 2004), CD44 (Morrison et al., 2001) and the two PDZ domain–containing adaptors EBP50/NHE-RF1 and E3KARP/NHE-RF2 (Murthy et al., 1998; Nguyen et al., 2001), the mechanism whereby Merlin controls cell proliferation remains poorly understood (McClatchey and Giovannini, 2005).

We have recently found that a signature of Nf2 deficiency in several types of primary cells, including both mesenchymal and epithelial cells, is a failure to undergo contact-dependent inhibition of proliferation and to establish stable cadherin-mediated adherens junctions (AJs) between cells (Lallemand et al., 2003). Merlin is regulated by cell–cell contact (Shaw et al., 1998), localizes to AJs, and physically associates with AJ components. Although core cadherin–catenin complexes are present in the membrane of *Nf2*$^{-/-}$ cells, stable AJ structures are not maintained. Defective AJs and loss of contact-dependent inhibition of proliferation may explain the tumorigenic and metastatic consequences of Nf2 deficiency. However, the nature of the mitogenic signals that drive proliferation of *Nf2*$^{-/-}$ cells with defective AJs is not known; indeed, the general mechanism of contact-mediated inhibition of proliferation is not well understood.

Here we present novel mechanistic insight into a critical role for the NF2 tumor suppressor, Merlin, in coordinating the processes of AJ stabilization with contact-dependent inhibition of epidermal growth factor receptor (EGFR) activity. In the

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Abbreviations used in this paper: AJ, adherens junction; EGFR, epidermal growth factor receptor; ERM, Ezrin/Radixin/Moesin; FERM, four-point-one, ERM; LDC, liver-derived epithelial cell; MEF, mouse embryo fibroblast; NF2, neurofibromatosis type 2; OB, osteoblast; pTyr, phosphotyrosine; RTK, receptor tyrosine kinase; Tr-EGF, Texas red–conjugated EGF.

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absence of Merlin, confluent cells are unable to silence mitogenic signaling from the EGFR, and their continuous proliferation is blocked by specific pharmacological inhibitors of the EGFR. Upon cell–cell contact, Merlin associates with EGFR via NHERF1 and prevents both ligand-induced EGFR internalization and the association of EGFR with its canonical effectors, precluding downstream signaling. Our data are consistent with a model whereby upon cell contact Merlin restrains EGFR into a membrane compartment from which it can neither signal nor be internalized. These studies reveal a novel mechanism of tumor suppressor function, linking the function of Merlin to that of a well-known oncogene and suggesting a possible therapeutic strategy for *NF2* mutant tumors.

Results

Cell density–dependent regulation of EGFR by Merlin

Our previous studies suggest that due to their inability to establish stable AJs, $Nf2^{-/-}$ cells continue to proliferate after reaching confluence. However, these studies do not reveal the source of mitogenic signals that drive the continued proliferation of confluent $Nf2^{-/-}$ cells—in essence the mechanism whereby Nf2-expressing cells normally undergo contact-dependent inhibition of proliferation. We could not detect altered β-catenin activity, nuclear localization, or changes in specific cadherin expression in these cells, suggesting that activated β-catenin does not drive the overproliferation of confluent $Nf2^{-/-}$ cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200703010/DC1; not depicted); in fact, normal levels of cadherin-associated β-catenin are present in the membranes of confluent $Nf2^{-/-}$ cells (Lallemand et al., 2003). Instead, confluent $Nf2^{-/-}$ cells exhibit sustained activation of signaling molecules that are not known β-catenin targets (Fig. 1 E).

Alternatively, accumulating evidence suggests that cadherin-dependent cell adhesion can control mitogenic signaling by negatively regulating receptor tyrosine kinases (RTKs) at the plasma membrane (Takahashi and Suzuki, 1996; Grazia Lampugnani et al., 2003; Qian et al., 2004). To determine whether Merlin function is important for contact-dependent inhibition of RTK signaling, we compared the profile of protein tyrosine phosphorylation in wild-type and $Nf2^{-/-}$ mouse embryo fibroblasts (MEFs) as they progress to high cell density with an increasing area of cell–cell contact in the presence of serum (see Fig. S2 A for a definition of confluence; available at http://www.jcb.org/cgi/content/full/jcb.200703010/DC1). As shown in Fig. 1 A, tyrosine phosphorylation of multiple proteins in a total membrane fraction was sharply down-regulated in confluent wild-type MEFs but not in confluent $Nf2^{-/-}$ MEFs. Upon serum deprivation, phosphotyrosine (pTyr) levels declined in both confluent wild-type and $Nf2^{-/-}$ membranes, indicating their dependence upon soluble growth factors (Fig. 1 A, right). Thus, in proliferating wild-type MEFs, serum growth factors maintain a physiological level of tyrosine kinase activity that is down-regulated as

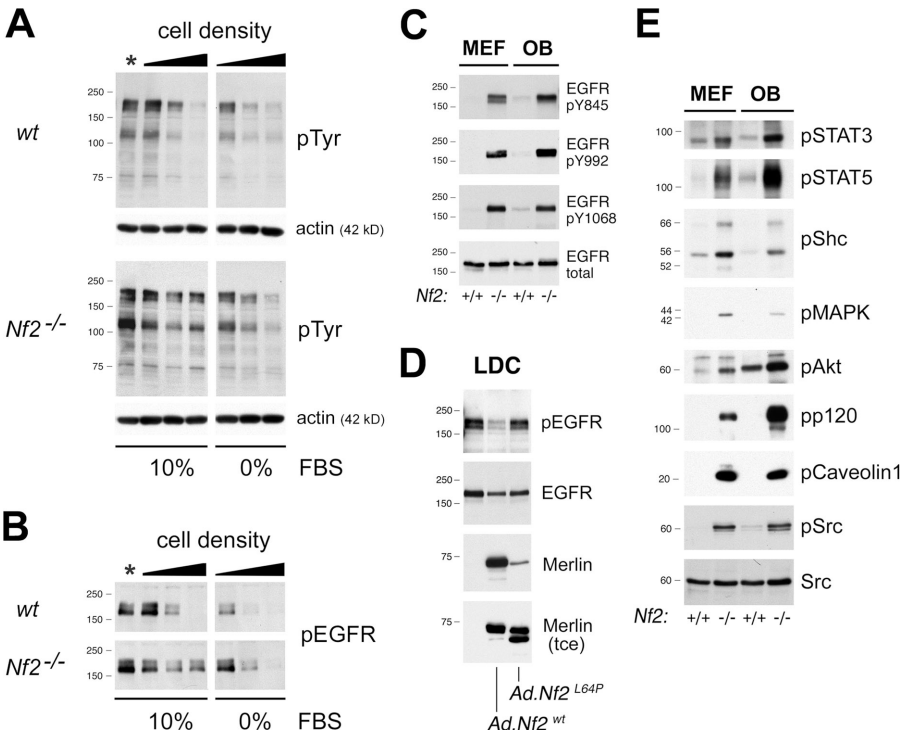

Figure 1. **Persistent EGFR signaling in multiple types of confluent $Nf2^{-/-}$ cells.** (A) Tyrosine-phosphorylated proteins in total membrane preparations from MEFs at increasing stages of confluence. At early confluence (*) in the presence of serum, wild-type (top) and $Nf2^{-/-}$ (bottom) cells display a similar pattern of pTyr; (see Fig. S2 for the definition of confluence). Progression to late confluence is accompanied by marked down-regulation of tyrosine kinase activity in wild-type, but not $Nf2^{-/-}$ cells (left). In contrast, both wild-type and $Nf2^{-/-}$ cells can down-regulate membrane pTyr after serum starvation (right). Actin = loading control. (B) EGFR activation, detected with an antibody against phosphorylated Y845, parallels the pTyr content observed in response to increasing confluence and serum starvation. In wild-type MEFs, pEGFR levels decrease as confluence progresses, but remain high in $Nf2^{-/-}$ cells in a serum-dependent manner. (C) Phosphorylation of multiple EGFR tyrosine residues is down-regulated in wild-type late-confluent primary MEFs and OBs grown in serum, but persist in the absence of Merlin. (D) Membranes of confluent $Nf2^{-/-}$ LDCs display high pEGFR levels that are markedly reduced upon adenoviral reintroduction of $Nf2^{wt}$, but not mutant $Nf2^{L64P}$. $Nf2^{L64P}$ is underrepresented in the membrane fraction, but comparable to that of $Nf2^{wt}$ in the total cell extract (tce). (E) Persistent activation of multiple EGFR targets is evident in membrane extracts from confluent $Nf2^{-/-}$ cells compared with wild-type. Phosphorylated active forms are indicated by the letter "p" preceding the name of the protein. All experiments were performed at least three times.

confluence progresses; this down-regulation is defective in the absence of Merlin.

Genetic cooperativity has been demonstrated between NF2 and EGFR pathway mutations in *Drosophila* (LaJeunesse et al., 2001). Moreover, the EGFR localizes to cell junctions, regulates cell adhesion, and can be negatively modulated by cadherin-dependent cell contact (Hoschuetzky et al., 1994; Takahashi and Suzuki, 1996; Pece and Gutkind, 2000; Betson et al., 2002; Qian et al., 2004). However, the basis of coordination between cell–cell contact and EGFR activity is not known. Given the prominent pTyr-containing protein of 170 kD (the molecular weight of EGFR) apparent in confluent $Nf2^{-/-}$ membranes (Fig. 1 A), we used antibodies against active, phosphorylated EGFR (pEGFR) to monitor EGFR activation in confluent wild-type and $Nf2^{-/-}$ cells. We found that the steady-state levels of active EGFR also diminished with increasing cell density in membranes of wild-type MEFs but persisted in $Nf2^{-/-}$ MEFs (Fig. 1 B).

To determine whether EGFR deregulation is a general signature of Nf2 deficiency, we examined EGFR activation in confluent primary osteoblasts (OBs) and liver-derived epithelial cells (LDCs), two key targets of *Nf2*-associated tumorigenesis in mice (McClatchey et al., 1998; Giovannini et al., 2000). Neither $Nf2^{-/-}$ OBs nor LDCs undergo contact-dependent inhibition of proliferation (Fig S2 B; see Fig. 6). Like MEFs, wild-type OBs down-regulate membrane pTyr and pEGFR at high cell density in the presence of serum, whereas $Nf2^{-/-}$ OBs maintain elevated levels of both (Fig. 1 C; not depicted). Similarly, confluent $Nf2^{-/-}$ LDCs retain high levels of pEGFR (Fig. 1 D). Reintroduction of wild-type Nf2 ($Nf2^{wt}$) into $Nf2^{-/-}$ MEFs, OBs, and LDCs restores contact-dependent inhibition of proliferation, electrondense AJs, and low levels of both pTyr and pEGFR (Fig. 1 D, Fig. 2 B; Fig. S2 C; not depicted). In contrast, a version of Merlin containing a patient-derived missense mutation ($Nf2^{L64P}$) fails to stably localize to AJs (Lallemand et al., 2003; Fig. 3 C), inhibit proliferation, or reduce pEGFR levels (Fig. 1 D). Multiple tyrosine residues on the EGFR and multiple EGFR targets remain phosphorylated in confluent $Nf2^{-/-}$ cells, suggesting that a program of EGFR signaling fails to be down-regulated (Gschwind et al., 2004) (Fig. 1, C and E). These data indicate that a continuous physiological activation of the EGFR persists in the membranes of confluent $Nf2^{-/-}$ cells; this is consistent with the fact that they do not proliferate faster than wild-type cells, but proliferate continuously despite reaching confluence. Thus, three different cell types all fail to undergo contact-dependent inhibition of proliferation and to down-regulate EGFR signaling at high cell density in the absence of Merlin.

Merlin controls the activity and membrane distribution of EGFR upon cell–cell contact

To understand how Merlin normally controls EGFR activity, we examined EGFR signaling as wild-type cells reach high density. Confluent epithelial cells of breast and kidney origin become unresponsive to stimulation by EGF and other growth factors via a cadherin-dependent mechanism (Takahashi and Suzuki, 1996; Qian et al., 2004). Accordingly, we found that while acute EGF stimulation of confluent Nf2-expressing cells does induce tyrosine phosphorylation of the receptor itself, activation of EGFR effectors such as Src and Raf does not increase, suggesting that signal propagation from the activated EGFR is prevented once wild-type cells reach high cell density (Fig. 2 A). In contrast, stimulation of confluent $Nf2^{-/-}$ cells results in a slight increase in the already elevated membrane phosphotyrosine content (Fig. 2 A). Reintroduction of $Nf2^{wt}$ but not $Nf2^{L64P}$ restored the block of EGFR signaling at high cell density (Fig. 2 B). Merlin does not appear to be a general inhibitor of RTK activation because signaling from the IGF-I receptor, including its ability

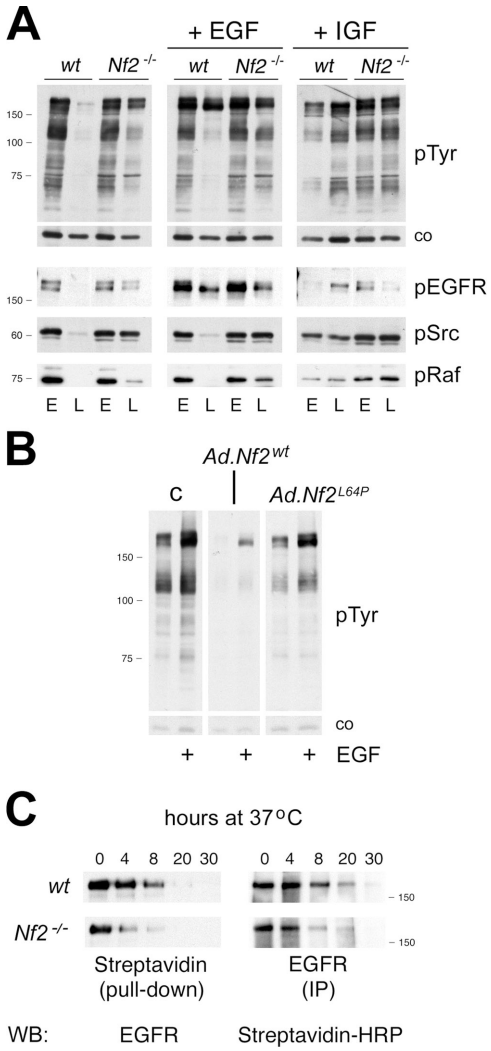

Figure 2. **Merlin prevents EGFR signaling in confluent cells.** (A) In late-confluent (L) wild-type MEFs, EGF-induced pTyr is limited to the EGFR without concomitant activation of EGFR downstream effectors such as Src and Raf. Increased membrane pTyr is already present in late-confluent $Nf2^{-/-}$ MEFs. The response to IGF-I is not prevented by Merlin. (EGF: 40 ng/ml, 30 min; IGF-I: 100 ng/ml, 30 min) (E = early confluence; co = loading controls). (B) Reintroduction of $Nf2^{wt}$ but not $Nf2^{L64P}$ abrogates persistent membrane pTyr in late-confluent $Nf2^{-/-}$ MEFs; as in wild-type MEFs, EGF stimulation of $Nf2^{wt}$-expressing MEFs affects pTyr of only the EGFR itself. (C) Surface EGFR turnover in late-confluent OBs. Confluent wild-type and $Nf2^{-/-}$ OBs were surface biotinylated at 4°C for 1 h and then shifted to 37°C in the presence of serum. At the indicated time points, the amount of biotinylated EGFR remaining in the cell was evaluated in both the total biotinylated fraction and in EGFR immunoprecipitates. Compared with wild-type OBs (top panels), $Nf2^{-/-}$ OBs (bottom panels) show accelerated clearing of surface-biotinylated EGFR. All experiments were performed at least three times.

to transactivate the EGFR (Roudabush et al., 2000), occurs in confluent MEFs regardless of the *Nf2* status (Fig. 2 A).

Induced phosphorylation of EGFR without activation of downstream targets in confluent, Nf2-expressing cells suggested that in this context the ability of the activated receptor to acquire signaling competence might be physically restricted by Merlin at cell confluence. Because Merlin is membrane associated and internalization of liganded EGFR is intimately linked to its signaling output (Sorkin and Von Zastrow, 2002), we asked whether the absence of Merlin had any effect on the surface levels of EGFR. We biotin-labeled cell surface proteins in confluent wild-type and $Nf2^{-/-}$ OBs. Although major differences in the levels of surface EGFR were not detected in wild-type and Nf2-expressing cells, clearance of EGFR was more rapid in confluent $Nf2^{-/-}$ cells, consistent with an increased rate of activation and internalization (Fig. 2 C).

Merlin prevents EGFR internalization and effector association in contacting cells

To further explore the role of Merlin in EGFR membrane localization we used Texas red–conjugated EGF (Tr-EGF) to visually track the EGFR in the presence and absence of Merlin. For these experiments we chose to use epithelial LDCs that are amenable to immunofluorescence localization analyses. In confluent $Nf2^{-/-}$ LDCs we found that within 30 min after stimulation Tr-EGF localizes to intracellular vesicles in virtually every cell, consistent with ligand-activated EGFR internalization (Fig. 3 A and Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200703010/DC1). We then generated mosaic cultures to compare $Nf2^{-/-}$ LDCs to neighboring cells into which $Nf2^{wt}$ or $Nf2^{L64P}$ were reintroduced. Tr-EGF internalization was prevented by expression of $Nf2^{wt}$ that, like EGFR, is enriched at cell–cell boundaries (Fig. 3 B and Fig. S3 B), but

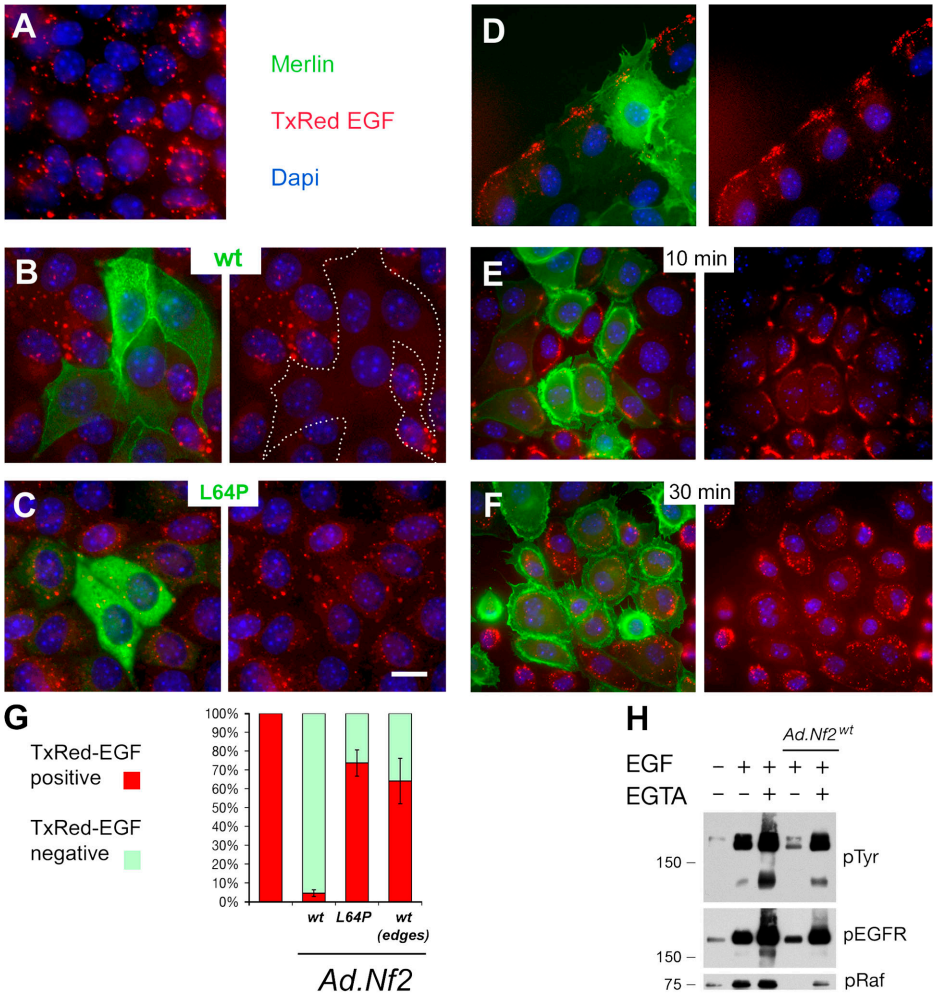

Figure 3. **Control of EGFR internalization and signaling by Merlin is contact dependent.** (A–F) Internalization of fluorescent Tr-EGF (2 μg/ml, 30 min) in confluent LDCs. (green = Merlin; red = Tr-EGF; blue = DAPI). (A) Tr-EGF containing vesicles are found within virtually every $Nf2^{-/-}$ LDC cell. (B) Internalized Tr-EGF is rarely observed in cells expressing wild-type Merlin. Dotted lines demarcate non-internalizing $Nf2^{wt}$-expressing cells. (C) Tr-EGF internalization is not prevented in cells expressing $Nf2^{L64P}$ or, (D) in cells that express $Nf2^{wt}$ but are situated at the free edge of a scrape wound. In these cells, Tr-EGF internalization occurs along the free edge itself. (E and F) Disruption of intercellular adhesion by Ca^{2+} depletion promotes Tr-EGF internalization in confluent $Nf2^{wt}$-expressing LDCs. E = 10 min; F = 30 min after Tr-EGF addition. Bar, 10 μm. (G) Average percentage distribution of ligand-internalizing cells in relation to Merlin expression and cell density. 200 $Nf2^{wt}$ or $Nf2^{L64P}$-expressing cells enclosed within a confluent monolayer, or $Nf2^{wt}$ cells situated at the free edges of nonconfluent cultures, were scored in each of four separate experiments. Cells with internalized Tr-EGF were designated as positive (error bars, ± SD). (H) Disruption of intercellular adhesion by Ca^{2+}-depletion restores EGFR signaling in $Nf2^{wt}$-expressing LDCs. EGF (40 ng/ml, 30 min) was added to starved LDCs after 30 min preincubation in EGTA/Ca^{2+}-free medium.

not by $Nf2^{L64P}$, which is excluded from cell–cell boundaries (Fig. 3 C; Lallemand et al., 2003). An identical response was seen after basolateral exposure to Tr-EGF (not depicted). Internalization of fluorescent transferrin proceeded similarly in the presence or absence of Merlin (Fig. S3 C). Importantly, $Nf2^{wt}$ did not prevent Tr-EGF internalization from the free edge of cells bordering a scrape wound or small colony (Fig. 3, D and G; not depicted), consistent with the hypothesis that Merlin limits EGFR internalization specifically upon cell–cell contact. In fact, disruption of cadherin-based intercellular adhesion by EGTA/Ca^{2+} depletion resulted in the appearance of internalized Tr-EGF (Fig. 3, E and F) and increased EGFR signaling in $Nf2^{wt}$-expressing cells (Fig. 3 H). Importantly, endogenous levels of Merlin also prevented EGFR internalization in similar mosaic cultures (Fig. S3 D).

These results suggest that upon cell contact Merlin functions to physically restrict ligand-activated EGFR from signaling. This interpretation is supported by the altered distribution of pEGFR in fractionated Triton-insoluble membranes in the absence of Merlin (Fig. 4 A). Although EGF stimulation of confluent wild-type cells yields the appearance of pEGFR that is confined to higher density fractions (II and III) that also contain Merlin, both pEGFR and Merlin are excluded from fraction I (Fig. 4 A). In contrast, upon EGF stimulation of confluent $Nf2^{-/-}$ cells, a substantial pool of pEGFR appears in the lowest density fraction (I; Fig. 4 A). These results suggest that the physical state of pEGFR is altered in the absence of Merlin. Merlin, EGFR, and AJ components are all normally enriched in the Triton-insoluble membrane fraction, a poorly defined biochemical compartment enriched in signaling molecules and cytoskeletal components and variously referred to as detergent-resistant membranes, lipid rafts, cholesterol-rich domains, etc. (Adams et al., 1996; Roepstorff et al., 2002; Lucero and Robbins, 2004; Stickney et al., 2004). Notably, the membrane distribution of Rac and RhoGDI, two proteins implicated in Merlin function, is unaffected by the absence of Merlin; in fact, in contrast to a recent report (Okada et al., 2005), we do detect recruitment of Rac to detergent-resistant membranes in both the presence and absence of Merlin (Fig. 4 A; not depicted). Consistent with this interpretation, reintroduction of $Nf2^{wt}$ but not $Nf2^{L64P}$ alters the solubility of EGFR in confluent LDCs (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200703010/DC1).

As shown in Figs. 1 and 2, the propagation of signaling from the activated EGFR to its downstream targets is blocked in confluent Nf2-expressing cells. Therefore, we asked whether Merlin directly interferes with the ability of ligand-activated EGFR to interact with its canonical signaling effectors. Consistent with established models of EGFR activation, EGF stimulation of confluent $Nf2^{-/-}$ LDCs causes EGFR to interact with Cbl, Grb2, Sos, and PLCγ; however, these interactions do not occur in the presence of Merlin (Fig. 4 B). Reintroduction of $Nf2^{wt}$, but not $Nf2^{L64P}$, prevents EGFR association with its immediate effectors in response to EGF despite phosphorylation of the EGFR itself (Fig. 4 B); in fact, we do not detect changes in the responsiveness of EGFR to EGF ligand (Fig. S4 B). Importantly, under these conditions wild-type Merlin, but not $Nf2^{L64P}$, physically associates with the EGFR (Fig. 4 B). Altogether, these data suggest that Merlin prevents EGFR from interacting with its immediate targets by sterically hindering the interaction and/or by sequestering the EGFR into a non-signaling membrane compartment from which both access to its downstream effectors and internalization are impeded. These data also indicate that Merlin acts at a step that precedes endocytosis of the activated EGFR. Indeed, EGF-induced Src activation and EGFR interaction with Grb2 and Cbl, early events that are required for EGFR internalization, do not occur in confluent Nf2-expressing cells (Wilde et al., 1999; Stang et al., 2004; Johannessen et al., 2006).

The PDZ domain–containing adaptor NHE-RF1 mediates Merlin–EGFR association

NHE-RF1 is a PDZ domain–containing adaptor that interacts with Merlin and the ERM proteins (Reczek et al., 1997; Murthy et al., 1998; for review see Bretscher et al., 2002) and is thought to play an important role in controlling the surface availability of certain membrane receptors including the β-adrenergic receptor and cystic fibrosis transmembrane conductance regulator (for review see Weinman et al., 2006). Importantly, recent studies indicate that NHE-RF1 can also interact with and alter the surface availability of the EGFR (Lazar et al., 2004). This raises

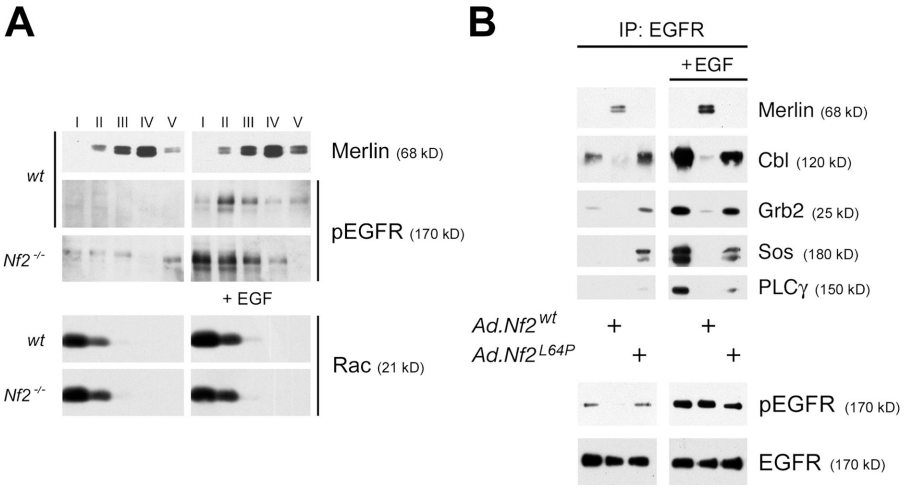

Figure 4. **Merlin controls EGFR membrane distribution and access to downstream effectors.** (A) Optiprep gradient fractionation of Triton-insoluble membranes from late-confluent wild-type and $Nf2^{-/-}$ MEFs reveals altered physical distribution of pEGFR in $Nf2^{-/-}$ cells after EGF stimulation (40 ng/ml, 30 min). In wild-type cells, pEGFR appears predominantly in fractions II and III, where Merlin is also present; both are excluded from the lowest density fraction I. In the absence of Merlin a unique pool of pEGFR appears in fraction I. In contrast, distribution of the small GTPase Rac is independent of Merlin status. (B) After reintroduction into $Nf2^{-/-}$ LDCs, $Nf2^{wt}$ but not $Nf2^{L64P}$ complexes with EGFR and prevents its interaction with multiple signaling effectors in response to EGF (40 ng/ml, 10 min). Experimental conditions are as in Fig. 1 D. All experiments were performed at least three times.

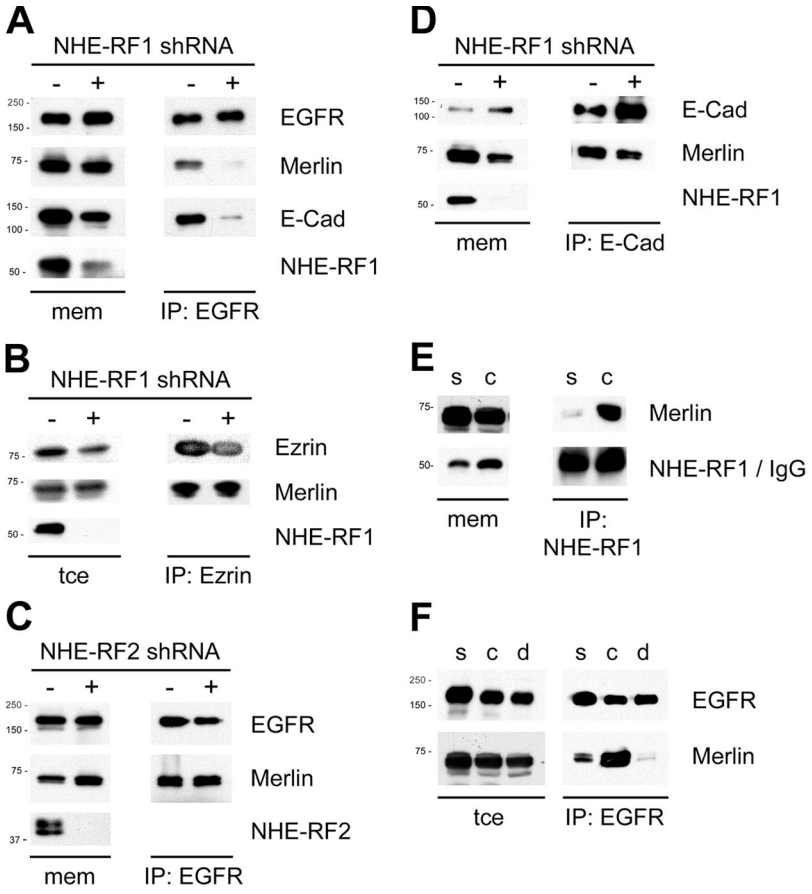

Figure 5. **NHE-RF1–mediated, contact-dependent association of Merlin and EGFR.** (A) Immunoprecipitation of EGFR from total membrane extracts of $Nf2^{wt}$-expressing LDCs with and without lentiviral NHE-RF1 shRNA expression revealed that the association of both $Nf2^{wt}$ and E-cadherin with the EGFR are nearly eliminated by NHE-RF1 knockdown. (B) Immunoprecipitation of Ezrin reveals that the association of $Nf2^{wt}$ with Ezrin occurs in a NHE-RF1–independent manner. (C) NHE-RF2 knockdown does not affect EGFR-Merlin association. (D) The association between $Nf2^{wt}$ and E-cadherin is not affected by NHE-RF1 down-regulation. (E) Immunoprecipitation of NHE-RF1 from total membrane extracts of subconfluent (s) and confluent (c) $Nf2^{wt}$-expressing LDCs revealed that the association between $Nf2^{wt}$ and NHE-RF1 occurs only in confluent conditions. Total IgG is shown as a control for the NHE-RF1 immunoprecipitations. (F) Immunoprecipitation of EGFR from total membrane extracts of $Nf2^{wt}$-expressing LDCs when sparse (s), confluent (c), and after cell–cell contact disruption by incubation in Ca^{2+}-depleted medium for 45 min (d). Complexing of Merlin and EGFR markedly increases from sparse to confluent cells and is abrogated by acute loss of intercellular contacts. All experiments were performed at least three times.

the possibility that Merlin regulates the surface availability of EGFR via NHE-RF1. To determine whether NHE-RF1 mediates the association between Merlin and EGFR, we performed shRNA-mediated knockdown of NHE-RF1 expression in $Nf2^{wt}$-expressing LDCs. Lentiviral expression of a shRNA targeting NHE-RF1 revealed that reduced NHE-RF1 expression nearly eliminated the association of $Nf2^{wt}$ and EGFR (Fig. 5 A). In contrast, $Nf2^{wt}$ associates with Ezrin regardless of the level of NHE-RF1 expression (Fig. 5 B). Importantly, NHE-RF2 does not detectably associate with EGFR in these cells and shRNA-mediated knockdown of NHE-RF2 expression has little effect on the association between Merlin and EGFR (Fig. 5 C; not depicted). These data suggest that Merlin–EGFR association is mediated specifically by NHE-RF1.

Adhesion-dependent function of Merlin

Merlin localizes to AJs and is required for AJ stabilization (Lallemand et al., 2003). Our previous studies suggest that upon cell–cell contact, Merlin is recruited to and activated at nascent AJs; indeed, Merlin also associates with E-cadherin in epithelial cells (Lallemand et al., 2003). The simplest interpretation of our data is that active, cadherin-associated Merlin "captures" the NHE-RF1–EGFR complex, thereby retaining it. Consistent with this hypothesis, we found that the association between EGFR and E-cadherin in confluent Nf2-expressing cells is NHE-RF1 dependent, whereas the association of Merlin with E-cadherin is NHE-RF1 independent (Fig. 5, A and D). A key prediction of this model is that the association between Merlin and both NHE-RF1 and EGFR is dependent on cell–cell contact; indeed, as shown in Fig. 5 (E and F), the association between Merlin and NHE-RF1 or EGFR is dramatically enhanced with increasing cell density. In contrast, the association between EGFR and NHE-RF1 is not adhesion dependent (Fig S5 A, available at http://www.jcb.org/cgi/content/full/jcb.200703010/DC1). Importantly, after acute disruption of intercellular contacts by Ca^{2+} depletion, Merlin rapidly dissociates from EGFR (Fig. 5 F), indicating that cell–cell adhesion is a strict determinant for EGFR–Merlin association.

Pharmacological inhibition of EGFR inhibits proliferation of confluent *Nf2*$^{-/-}$ cells

To determine whether EGFR activation is responsible for the persistent tyrosine phosphorylation of membrane proteins and proliferation of confluent $Nf2^{-/-}$ cells, we treated $Nf2^{-/-}$ MEFs, OBs, and LDCs with pharmacologic EGFR inhibitors. Both Compound 56 and Gefitinib (Iressa), potent specific inhibitors of EGFR kinase activity (Gschwind et al., 2004), eliminated the high membrane pTyr content in confluent $Nf2^{-/-}$ cells of all three cell types in the presence of serum (Fig. 6 A; not depicted). The specificity of each compound was demonstrated by its ability to block EGF- but not PDGF-induced membrane pTyr (Fig. 6 A; not depicted). Importantly, before confluence, EGFR inhibitors had only a modest effect on the proliferation of primary $Nf2^{-/-}$ MEFs, OBs, and LDCs in the presence of serum

(Fig. 6, B and C; not depicted), indicating that proliferation of non-contacting cells can be sustained by serum-derived signals other than those mediated by EGFR. However, EGFR inhibition halted proliferation at high cell density; thus, *Nf2*$^{-/-}$ cells can undergo contact-dependent inhibition of proliferation if EGFR activity is blocked. As shown in Fig. 6 D, EGFR inhibition did not restore permanent contact-dependent inhibition of proliferation to *Nf2*$^{-/-}$ cells because withdrawal of the inhibitor led to reentry into the proliferative state despite the high cell density. These results suggest that EGFR inhibitors, already in clinical use for several human cancers, could also be of therapeutic benefit for NF2-deficient tumors. In fact, EGFR inhibitors may actually be more efficacious in preventing the physiologic EGFR activation that persists in confluent *Nf2*$^{-/-}$ cells rather than the high levels driven by oncogenic EGFR mutations. Notably, signaling via EGFR family members (ErbBs) is critical for the proliferation and survival of Schwann cells, the principle target of NF2-associated tumorigenesis in humans (Garratt et al., 2000).

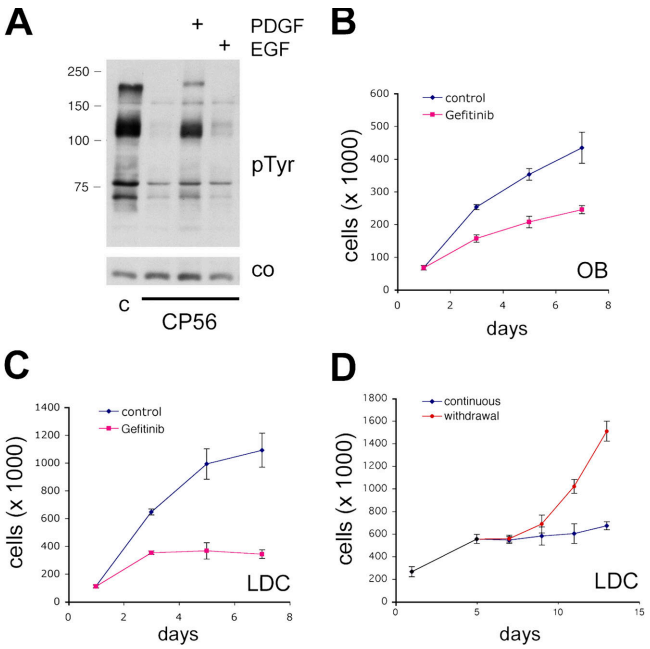

Figure 6. **Pharmacological inhibitors of EGFR arrest proliferation of *Nf2*$^{-/-}$ cells.** (A) Tyrosine phosphorylation of membrane proteins in late-confluent *Nf2*$^{-/-}$ MEFs is eliminated by the EGFR inhibitor Compound 56. In the presence of the inhibitor (added 2 h before EGF addition), pTyr induction is blocked after stimulation with EGF (40 ng/ml) but not PDGF (35 ng/ml) for 30 min. Similar specificity was observed for Gefitinib (not depicted). Proliferation of *Nf2*$^{-/-}$ OBs (B) and LDCs (C) at high cell density is inhibited by Gefinitib. (D) Inhibition of EGFR by Gefitinib does not permanently restore contact inhibition of growth in LDCs. All experiments were performed at least three times. (Error bars, ± SD).

Discussion

The discovery, in 1993, that the NF2 tumor suppressor, Merlin, is a member of a family of membrane/cytoskeleton-associated proteins suggested a novel mechanism of tumor suppression (Rouleau et al., 1993; Trofatter et al., 1993). Amidst the identification of many Merlin-interacting proteins and Merlin-controlled activities, a clear role for Merlin in controlling contact-dependent inhibition of proliferation has emerged (Morrison et al., 2001; Johnson et al., 2002; Lallemand et al., 2003). Loss of contact-dependent inhibition of proliferation is a signature of cell transformation, but the molecular basis of this phenomenon is not known. Our previous work identified a role for Merlin in stabilizing AJs between cells, but did not pinpoint the mitogenic signal that drives proliferation in the absence of Merlin and normal AJs (Lallemand et al., 2003). An intimate relationship between RTK activity and AJ stability clearly exists, but its molecular underpinnings are only beginning to emerge (for review see Brunton et al., 2004). We have now found that Merlin directly interferes with EGFR signaling in a contact-dependent manner, providing key insight into the molecular basis of contact-dependent inhibition of proliferation and directly linking the functions of a novel tumor suppressor and a well-known oncogene.

The aberrant cell–cell communication and persistent EGFR signaling in confluent *Nf2*$^{-/-}$ cells, together with the localization of EGFR to AJs, suggests that Merlin normally coordinates the processes of AJ stabilization and negative regulation of the EGFR by establishing their interdependence as they occur. Our data are consistent with a model wherein the following sequence of events occurs (Fig. 7 A): Merlin is recruited to nascent AJs (Lallemand et al., 2003) where it is activated and begins to stabilize the developing junctions and sequester a pool of EGFR into a compartment from which it does not have access to its immediate downstream targets and cannot be internalized. Whether Merlin is in an "open" or "closed" conformation at this stage remains to be established; although many studies suggest that "active" Merlin is not phosphorylated at serine 518, it is not clear whether this hypophosphorylated form of Merlin is consistently self-associated. The ensuing localized reduction of EGFR tyrosine kinase activity at contact sites, in turn, may further stabilize AJs, perhaps via altering the phosphorylation of AJ components such as p120 (see Fig. 1 E) and/or the activity of Rac. Indeed, while initial stages of AJ formation are accompanied by increased tyrosine kinase activity and activation of the small GTPase Rac, a classic target of EGFR signaling that mediates dynamic membrane/cytoskeletal remodelling at cell–cell interfaces (Pece and Gutkind, 2000; Betson et al., 2002; Goodwin et al., 2003), later stages are often associated with negative regulation of both EGFR and Rac (Brunton et al., 2004). This could explain previously reported roles for Merlin in negatively regulating both Rac and its effector Pak and in modulating actin cytoskeleton remodelling (James et al., 2001; Shaw et al., 2001; Kissil et al., 2003; Manchanda et al., 2005). Although the precise mechanism whereby Merlin associates with the actin cytoskeleton is not yet clear, we have found that association with the cortical actin cytoskeleton is necessary for the growth-suppressing and EGFR-inhibiting activities of Merlin (Cole, B.K., personal communication).

Molecularly, our studies indicate that Merlin directly links the AJ and EGFR. Indeed, the trilobed structure of the four-point-one, ERM (FERM) domain appears well-designed for assembling multiple proteins (Pearson et al., 2000). We found that the association between Merlin and EGFR is mediated by the tandem PDZ domain–containing adaptor NHE-RF1, which is known to interact with the third lobe of the ERM FERM domain

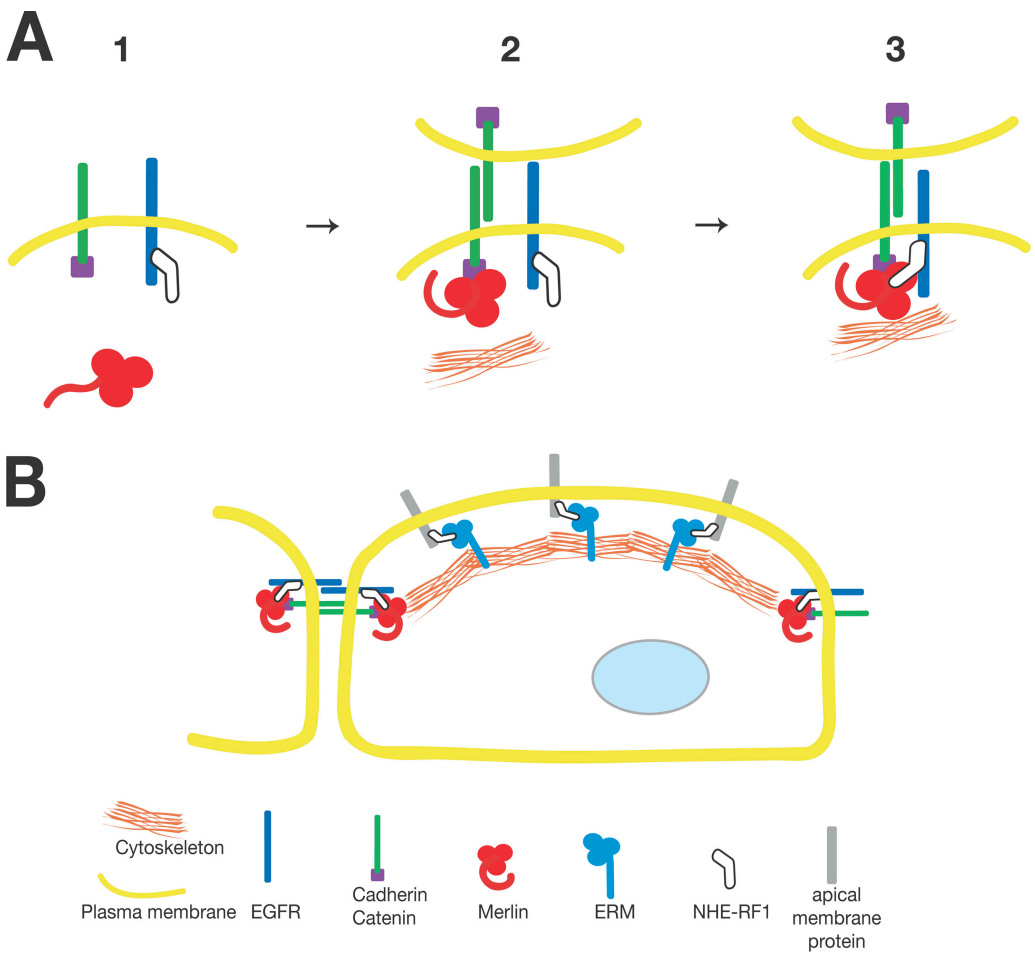

Figure 7. **Molecular and spatial attributes of Merlin function.** (A) Model depicting the mechanism of Merlin-mediated coordination of AJ stabilization and EGFR down-regulation. In non-contacting cells (1), Merlin does not associate with cadherin or EGFR; however, upon cell–cell contact (2), Merlin is recruited to nascent junctions and activated, stabilizing the interaction between junctional proteins and the cortical cytoskeleton. Activated Merlin then "captures" NHE-RF1-associated EGFR (3), preventing it from internalizing or signaling. Active Merlin is likely hypophosphorylated and could be in an "open" or "closed" (self-associated) conformation; indeed, more than two conformational states of Merlin may exist. Although Merlin lacks the C-terminal actin-binding domain present in the ERM proteins, some studies suggest that Merlin can interact directly with filamentous actin via the FERM domain. Despite an abundance of evidence linking Merlin physically and functionally to the actin cytoskeleton, the precise mechanism whereby Merlin associates with the actin cytoskeleton remains unclear. (B) Although Merlin and the ERM proteins can all interact with NHE-RF1 and NHE-RF1 can, in turn, interact with several membrane receptors, Merlin may be functionally dedicated to the junctional domain and the ERM proteins to the apical domain. In this way, spatial and temporal regulation of NHE-RF1–associated receptors may be achieved.

(Finnerty et al., 2004; Terawaki et al., 2006). NHE-RF1 can associate with and is thought to regulate the surface abundance of several different receptors (for review see Weinman et al., 2006). In fact, it has been reported that NHE-RF1 stabilizes and slows the down-regulation of surface EGFR (Lazar et al., 2004); however, neither this nor other studies have explored how functional specificity is applied to such a wide range of receptor interactions, and it is not clear how temporal and spatial regulation of NHE-RF1–associated receptors is achieved. Our studies suggest that Merlin confers one such level of specificity by locally engaging NHE-RF1–associated EGFR at the AJ.

The ERM proteins also interact with NHE-RF1 and are required for stable apical localization of NHE-RF1 in the intestinal epithelium (Saotome et al., 2004). However, the ERM proteins likely engage a distinct subset of NHE-RF–associated receptors. Consistent with the tendencies of the ERM proteins and Merlin to be apically or apical-junctionally concentrated, respectively, the ERM proteins appear to be functionally dedicated to the apical membrane and Merlin to the junctional domain (Fig. 7 B). By analogy to the roles of Merlin in both stabilizing the association between adhesion proteins and the actin cytoskeleton and locally "capturing" NHE-RF1–EGFR complexes, Ezrin may stabilize the association between the apical membrane and cortical cytoskeleton while capturing apical NHE-RF receptor complexes (Fig. 7 B). Indeed, Ezrin is required for establishing or maintaining the integrity of the apical surface of intestinal epithelial cells in vivo (Saotome et al., 2004). In contrast to Merlin, Ezrin does not associate with EGFR or E-cadherin, mediate contact-dependent inhibition of proliferation, or effect EGFR internalization/signaling in the cells studied here (Fig. S5 B), and overproliferation is not detected in the ERM-deficient intestine (Saotome et al., 2004).

It is clear that AJs are continuously remodelled both in confluent monolayers in culture and in tissues in vivo. In vivo,

all cells in solid tissues are in contact and junctional remodeling and cell proliferation must be exquisitely coordinated. Localization to intercellular adhesions may render the EGFR uniquely able to sense and modulate changes in cell contact and to fine-tune its activity accordingly. Our molecular model of Merlin-mediated coordination of AJ stabilization and EGFR down-regulation provides ample opportunity for flexibility. For example, phosphorylation or phospholipid binding may alter Merlin self-association and/or membrane distribution, coordinately affecting junction stability and EGFR signaling. Indeed, both hypo- and hyperphosphorylated forms of Merlin are associated with EGFR (Fig. 4 B, Fig. 5 A), suggesting that S518 phosphorylation may regulate Merlin-associated EGFRs. We found that the association of Merlin with E-cadherin and with NHE-RF1–EGFR are both contact dependent; however, it is interesting to note that while the association of Merlin with E-cadherin and NHE-RF1 is maintained after acute disruption of intercellular contacts (not depicted), the association between Merlin and EGFR is rapidly lost, suggesting disengagement of NHE-RF1–EGFR in this specific context. Our studies also indicate that the status of cell–cell contact has profound implications for the propagation of EGFR signaling. Conversely, in the context of EGFR-driven tumorigenesis, a critical line of investigation will be to determine whether oncogenic variants of the EGFR can evade the contact-dependent inhibition of signaling imposed by Merlin.

Pharmacologic EGFR inhibition abolished the persistent tyrosine phosphorylation of membrane proteins and the proliferation of three types of *Nf2*$^{-/-}$ cells in the presence of serum, suggesting that EGFR activation is necessary and sufficient to cause these phenotypes. This also suggests a novel avenue of therapeutic exploration for NF2. However, given that NHE-RF1 can associate with multiple receptors, Merlin may well affect other receptors by a similar mechanism. In fact, the results presented here are complementary to those of Maitra et al. (2006), who reported altered surface availability of EGFR and other membrane receptors in *Drosophila* tissues lacking both Merlin and the related tumor suppressor, Expanded. Similarly, Merlin could coordinate regulation of EGFR or other receptors with alternative adhesion receptors such as CD44; it has been proposed that Merlin mediates contact-dependent inhibition of proliferation via CD44 in other cell types (Morrison et al., 2001). Indeed, the theme of Merlin-mediated coordination of cell adhesion and membrane receptor signaling is echoed by recent work in *Drosophila* suggesting that Merlin inhibits signaling through the Hippo/Warts/Yorkie pathway (Huang et al., 2005; Hamaratoglu et al., 2006), corresponding to the conserved Mst/Lats/Yap pathway in mammals. Activation of this pathway in response to extracellular signals appears to be coordinately regulated by Merlin and Expanded, which signals from the Fat cadherin receptor (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006). However, neither the source of that extracellular signal nor the signaling receptor(s) involved have been identified in mammals or flies (Edgar, 2006). The data presented here indicate that Merlin could regulate signaling through this pathway by directly coordinating EGFR signaling output with cadherin-dependent intercellular adhesion.

Materials and methods

Cell culture and expression vectors

Wild-type and *Nf2*$^{-/-}$ primary MEFs were prepared as described previously (Lallemand et al., 2003). Wild-type primary OBs were prepared from calvaria of *Nf2*$^{flox2/flox2}$ newborn mice as described previously (Ducy and Karsenty, 1995). *Nf2* deletion in OBs was achieved via adenoviral expression of the Cre-recombinase as we described for MEFs (Lallemand et al., 2003). Primary MEFs and OBs were used between passages 3 and 6. Epithelial *Nf2*$^{-/-}$ LDCs were derived by liver-specific, deletion of *Nf2* in vivo by crossing *Nf2*$^{flox2/flox2}$ mice to transgenic *Alb-Cre* mice (Postic et al., 1999) (*B6.Cg-Tg(Alb-cre)21Mgn/J*; Jackson Laboratories). In brief, the liver of a 12-wk-old *Alb-Cre;Nf2*$^{flox2/flox2}$ mouse was removed, minced, dissociated in Liver Dissociation Medium (Invitrogen), and cultured in 10% FBS-DME. Wild-type epithelial embryonic liver cells were derived from the liver of a day-14.5 *Nf2*$^{flox2/flox2}$ embryo as described by Strick-Marchand and Weiss (2002), and subsequently adapted to the standard growth conditions used for all other cell lines. Clonal cell lines were established by limiting dilution. The generation and use of adenoviral vectors expressing Nf2^{wt} and Nf2^{L64P} have been described previously (Lallemand et al., 2003).

Antibodies

Primary antibodies against the following antigens were from Upstate Biotechnology (active-β-catenin: ABC, 05–665, 1:1,000 dilution); Transduction Laboratories (pTyr: RC20, 610023; β-catenin: 610153; E-cadherin: 610182; p120ctn: 610133; p120ctn-pY228: 612536; Caveolin-pY14: 611338; c-Cbl: 610441; Grb2: 610111; Sos1: 610095; PLCγ: 610027; Rac1: 612652; all at 1:1,000 or 2,000 dilution); Santa Cruz Biotechnology, Inc. (Merlin: sc331, 1:40,000 dilution; EGFR: sc1005); Cell Signaling (EGFR-pY845: 2231; EGFR-pY992: 2235; EGFR-pY1068: 2234; STAT3-pY705: 9131; STAT5-pY694: 9351; Shc-pY239/240: 2434; MAPK-pT202/Y204: 5120; AKT-pS473: 9271; Raf-pS259: 9421, all at 1:1,000 dilution); Biosource International (Src: 44–656; Src-pY418: 44–660, used at 1:1,000); Abcam (EBP50/NHERF-I: ab3452); NeoMarker (EGFR: Ab17; Ezrin: Ab1); Sigma-Aldrich (actin: A-2547). Monoclonal anti-Merlin 1C4 (a gift of Vijaya Ramesh, Massachusetts General Hospital, Boston, MA) was used at 1:1,000. Polyclonal anti-NHE-RF2 (B70; gift of Anthony Bretscher, Cornell University, Ithaca, NY) was used at 1:1,000. HRP-conjugated secondary antibodies to rabbit, mouse, or rat were from GE Healthcare.

Subcellular fractionation and Western blot analysis

Equal protein amounts of total cell and membrane extracts were analyzed by Western blot as described previously (Lallemand et al., 2003) with one modification: the membrane pellet was directly solubilized in RIPA buffer containing 0.5% SDS. For density-gradient separation, postnuclear membrane pellets from three 150-mm dishes of late confluent MEFs were lysed on ice for 30 min in Triton-lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 10 mM β-glycerophosphate plus protease and phosphatase inhibitors), resuspended in buffer A (250 mM sucrose, 1 mM EDTA, 20 mM tricine, and 1% Triton X-100 and inhibitors) containing 35% Optiprep, sequentially overlayed with 25, 15, 5, and 0% Optiprep-buffer A and centrifuged for 3.5 h at 160000 *g*. Fractions collected at the interfaces I = 0–5%, II = 5–15%, III = 15–25%, IV = 25–35%, and the pellet V > 35%, were resuspended in RIPA buffer and analyzed (7 μg/lane) by immunoblotting.

Immunofluorescence and ligand-internalization assay

LDCs plated on glass coverslips were infected with Ad.Nf2^{wt} or Ad.Nf2^{L64P} when ∼50% confluent. After 4–5 d, confluent monolayers were serum starved in 1% BSA in DME for 2 h, incubated for 30 min at 37°C with 2 μg/ml Tr-EGF (E3480; Molecular Probes) or 10 μg/ml Alexa Fluor 488-Transferrin (T13342; Molecular Probes), and fixed in 4% PFA-cytoskeletal buffer (10 mM MES, pH 6.3, 2 mM EGTA, 3 mM $MgCl_2$, and 138 mM KCl) for 15 min at room temperature. After permeabilization in 0.2% Triton X-100, cells were incubated with primary anti-NF2 antibody (sc331; 1:300 in 1% BSA-PBS) overnight at 4°C. After incubation with FITC- or rhodamine-conjugated anti–rabbit secondary antibody (Jackson Immunoresearch Laboratories; 1:200), coverslips were mounted with Vectashield (Vector Laboratories). To create noncontacting free edges in Ad.Nf2^{wt}-infected LDCs, monolayers growing in 10% FBS-DME were scrape-wounded with a pipet tip, allowed to recover for ∼6 h, and starved (2 h) before adding Tr-EGF as described above. To disrupt intercellular adhesion by depletion of extracellular Ca^{2+}, monolayers were serum starved for 2 h, washed twice in Ca^{2+}-free DME (Invitrogen), incubated in 2 μg/ml Tr-EGF, 1% BSA, and 5 mM EGTA in Ca^{2+}-free DME and fixed as above at the indicated

time points. Images were acquired using a 63× 1.4NA oil objective lens (Carl Zeiss MicroImaging, Inc.) on an Axioplan microscope (Carl Zeiss MicroImaging, Inc.) with IP Lab software and a Sony CCD camera. Final images were prepared using Adobe Photoshop 7.0.

Surface biotinylation and immunoprecipitation

Late confluent MEFs or OBs were serum starved overnight in DME, shifted to 4°C, rinsed twice in cold PBS and incubated for 1 h with 0.5 μg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemical Co.) in PBS. After quenching the reaction (50 mM NH_4Cl, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ for 10 min) and rinsing in PBS, cells were returned to 10% FBS-DME at 37°C and lysed in Triton-lysis buffer containing 60 mM Octylglucoside (*n*-Octyl-β-D-glucopyranoside; Calbiochem) at the indicated time points. Normalized extracts (600 μg total protein/400 μl) were precleared with protein A–Sepharose prebound to normal rabbit IgG for 2 h at 4°C. Anti-EGFR antibody (Ab17, NeoMarker; 8 μg/sample) or streptavidin-coupled agarose beads (50 μl; Pierce Chemical Co.) were added to precleared extracts and incubated overnight at 4°C. The following day, EGFR-containing immunocomplexes were precipitated with protein A–Sepharose beads (40 μl, 2 h, at 4°C). Beads from either immunoprecipitation or biotin pull-down were washed five times in the above buffer and boiled 5 min in 2× sample buffer. Complexes were separated by 8% SDS-PAGE and analyzed by Western blot as described above. Biotinylated-immunoprecipitated EGFR was detected with HRP-conjugated streptavidin. Immunoprecipitations of EGFR (Ab17; 8 μg) and E-cadherin (3 μg) from LDCs were from total membrane extracts in the above Triton-Octylglucoside buffer (800 μg total protein/400 μl). To disrupt intercellular adhesion by depletion of extracellular Ca^{2+}, confluent monolayers were washed twice in PBS/5 mM EGTA, and incubated in Ca^{2+}-free DME (Invitrogen) containing 10% Ca^{2+}-chelated FBS for 45 min.

Cell proliferation

MEFs, OBs (5×10^4) or LDCs (7.5×10^4) were seeded in triplicate 15-mm wells in 5% FBS-DME. The following day, 1 μM Gefitinib (Iressa; AstraZeneca) or 0.5 μM Compound 56 (Calbiochem) were added to culture wells; cells were trypsinized and counted every other day. Fresh medium with or without inhibitors was added each day of counting. For drug withdrawal, 1 μM Gefitinib was added to LDCs daily until day 5 post-seeding, when half of the wells were returned to 5% FBS-DME only. Beginning that day, cells were counted and fresh medium with or without inhibitor was added to the remaining wells every other day.

shRNA-mediated knockdown

Several shRNA constructs against NHE-RF1 (#68583-68587) and NHE-RF2 (#68613-68615, 68617) in the lentiviral pLKO.1 vector were obtained from Open Biosystems and tested for NHE-RF1/2 knockdown in $Nf2^{-/-}$ LDCs; #68587 and #68617 were used for experiments. Lentiviral production and infection was performed as described previously (Bailey et al., 2006).

Online supplemental material

Fig. S1 shows level and distribution of adhesion molecules in various cell types used in this work. Fig. S2 defines confluence states in mesenchymal cells and shows transmission electron micrographs that reveal restoration of electrondense AJs upon expression of $Nf2^{wt}$ in LDCs. Fig. S3 shows internalization of fluorescent EGF and transferrin in LDCs, EGFR localization in LDCs, and inhibition of fluorescent EGF internalization in confluent embryonic liver cells expressing endogenous *Nf2*. Fig. S4 shows decreased EGFR solubility in the presence of Merlin and a similar dose dependency of EGFR auto-phosphorylation in the presence or absence of Merlin. Fig. S5 shows that the association of endogenous EGFR and NHE-RF1 is contact independent and reveals the lack of association between Ezrin and EGFR or Ezrin and E-cadherin in LDCs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200703010/DC1.

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