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 (McCleathey and Giovannini, 2005). Loss of NF2 function is associated with the development of multiple cancers in humans and mice (McCleathey 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 (McCleathey 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
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 NHE-RF1 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.2007030310/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.2007030310/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, phosphorytrosine (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...
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 Nf2 phosphorylated in confluent Nf2−/− membranes 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.

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 (Nf2wt) into Nf2−/− MEFs, OBs, and LDCs restores contact-dependent inhibition of proliferation, electron-dense 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 Nf2wt 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 to drive proliferation and to down-regulate EGFR signaling at high cell density.
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 Nf2wt or Nf2L64P were reintroduced. Tr-EGF internalization was prevented by expression of Nf2wt 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 Nf2wt-expressing cells. (C) Tr-EGF internalization is not prevented in cells expressing Nf2L64P or (D) in cells that express Nf2wt 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 Ca2+ depletion promotes Tr-EGF internalization in confluent Nf2wt-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 Nf2wt or Nf2L64P-expressing cells enclosed within a confluent monolayer, or Nf2wt 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 Ca2+ depletion restores EGFR signaling in Nf2wt-expressing LDCs. EGF (40 ng/ml, 30 min) was added to starved LDCs after 30 min preincubation in EGTA/Ca2+-free medium.
not by Nf2<sup>−/−</sup>, 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<sup>wt</sup> 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<sup>2+</sup> depletion resulted in the appearance of internalized Tr-EGF (Fig. 3, E and F) and increased EGFR signaling in Nf2<sup>−/−</sup>-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<sup>−/−</sup> 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<sup>wt</sup> but not Nf2<sup>L64P</sup> 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<sup>−/−</sup>-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<sup>wt</sup>, but not Nf2<sup>L64P</sup>, 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<sup>L64P</sup>, 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...
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 Nf2wt-expressing LDCs. Lentiviral expression of a shRNA targeting NHE-RF1 revealed that reduced NHE-RF1 expression nearly eliminated the association of Nf2wt and EGFR (Fig. 5 A). In contrast, Nf2wt 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 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 Ca²⁺ 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.
associated tumorigenesis in humans (Garratt et al., 2000) and survival of Schwann cells, the principle target of NF2-EGFR family members (ErbBs) is critical for the proliferation driven by oncogenic EGFR mutations. Notably, signaling via NF2-deficient tumors. In fact, EGFR inhibitors may actually be more efficacious in preventing the physiologic EGFR activation that persists in confluent N2/− 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).

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 Merlin and 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 N2/− 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 further stabilizes AJs, perhaps via altering the phosphorylation 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 Merlin and 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.

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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.
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 S18 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 N2−/− 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 N2−/− primary MEFs were prepared as described previously (allemand et al., 2003). Wild-type primary OBs were prepared from calvaria of N2−/−embryos as described previously (Ducy and Karsenty, 1995). N2− deletion in OBs was achieved via adenaloviral expression of the Cre-recombinase as we described for MEFs (allemand et al., 2003). Primary hOBs and OBs were used between passages 3 and 6. Epithelial N2−/−LDCs were derived by liver-specific, deletion of N2I in vivo by crossing N2Iembryo to transgenic Alb-Cre mice (Pastic et al., 1999; B6.Cg-Talb-cre;121Mgn/J; Jackson Laboratories). In brief, the liver of a 12-wk-old Alb-Cre/N2I−/embryos was removed, minced, dissociated in Liver Dissection Medium (Invitrogen), and cultured in 10% FBS-DME. Wild-type epithelial embryonic liver cells were derived from the liver of a day 13.5 N2I−/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 N2I−/and N2I−/− have been described previously (allemand 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 (pyr: RC20, 610023; β-catenin: 610153; E-cadherin: 610182; p120ctn: 610133; p120ctn-pY228: 612536; Cavolinyl:4: 611338; cCbl: 610441; Grb2: 611011; Sosr: 610905; PLYC: 610027; Rac1: 612652; all at 1:1,000 or 2,000 dilution); Santa Cruz Biotechnology (Merlin: sc331, 1:40,000 dilution; EGFR: sc1005); Cell Signaling Technology (EGFR-pY845: 2231; EGFR-pY992: 2225; EGFR-pY116; 2283; STAT3-pY705: 9131; STAT3-pY694: 9351; SHC/pY239/240: 2434; MAPK-pT202/Y204: 5120; AKT-pS473/472; pAkt: 4729; all at 1:1,000 dilution); Biosource International [Src: 44–656, Src-pY418: 44–660, used at 1:1,000]; Abcam (EP50/HerF-I: ab3452); Neomarker (EGFR: Ab17; ErbB: Ab1); Sigma-Aldrich (actin: A-2547); Monoclonal anti-Merlin 1C4 (a gift of Vijayi Ramesh, Massachusetts General Hospital, Boston, MA) was used at 1:1,000. Polyclonal anti-NHERF2 (B70; gift of Anthony Bretsch, 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 (allemand 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 by Western blot as described previously (Lallemand et al., 2003). The membrane pellet was directly solubilized in RIPA buffer containing 0.5% SDS. For density-gradient separation, postnuclear membrane pellets were solubilized in detergent-containing buffer (10 mM MES, pH 6.3, 2 mM EGTA, 3 mM MgCl2, 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; [a gift of Vijayi Ramesh, Massachusetts General Hospital, Boston, MA]) at 1:1,000. Polyclonal anti-NHERF2 (B70; gift of Anthony Bretsch, Cornell University, Ithaca, NY) was used at 1:1,000. HRP-conjugated secondary antibodies to rabbit, mouse, or rat were from GE Healthcare.

Immunofluorescence and ligand internalization assay

LDCs plated on glass coverslips were infected with Ad.N2I−/ or Ad.N2I−/− when ~50–70% confluent. After ~4–5 d, confluent monolayers were serum starved in 1% BSA in DMEM for 2 h, incubated for 30 min at 37°C with 2% Tr-EGF (E3480; Molecular Probes) or 10 μg/ml Alexa Fluor 488-Transferin (T13342; Molecular Probes), and fixed in 4% PFA-cytoskeletal buffer (10 mM MES, pH 6.3, 2 mM EGTA, 3 mM MgCl2, 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.N2I−/infected LDCs, monolayers growing in 10% FBS-DMEM were scrape-wounded with a pipet tip, allowed to recover for ~6 h, and stained (2 h) before adding Tr-EGF as described above. To disrupt intercellular adhesion by depletion of extracellular Ca2+, monolayers were serum starved for 2 h, washed twice in Ca2+−/−free DMEM (Invitrogen), incubated in 2 μg/ml Tr-EGF, 1% BSA, and 5 mM EGTA in Ca2+−/−free DMEM and fixed as described above at the indicated.
time points. Images were acquired using a 63× 1.4NA oil objective lens (Carl Zeiss Microlamaging, Inc.) on an Axiosplan microscope (Carl Zeiss Microlamaging, 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 NaH2Cit, 1 mM MgCl2, and 0.1 mM CaCl2 for 10 min) and rinsing in PBS, cells were returned to 10% FBS-DME at 37°C and lysed in Triton-X buffer containing 60 μg/ml Ostarylglucoside (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; Neo-Marker; 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-Ostarylglucoside buffer (800 μg total protein/400 μl). To disrupt intercellular adhesion by depletion of extracellular Ca2+, confluent monolayers were washed twice in PBS/5 mM EGTA, and incubated in Ca2+-free DME (Invitrogen) containing 10% Ca2+-chelated FBS for 45 min.

Cell proliferation

MEFs, OBs (5 × 104) or LDCs (7.5 × 104) were seeded in triplicate 15-mm wells in 5% FBS-DME. The following day, 1 μM Gefitinib (Iressa; AstraZeneca) or 0.5 μM Compound S6 (Calbiochem) were added to culture wells; cells were trypsinized and counted each 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 N2+/− 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 describes confluence states in mesenchymal cells and shows transmission electron micrographs that reveal restoration of electron-dense adjs upon expression of N2+ 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 N2. Fig. S4 shows decreased EGFR solubility in the presence of Merlin and a similar dose dependence 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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