Regulation of PTEN/Akt and MAP kinase signaling pathways by the ubiquitin ligase activators Ndfip1 and Ndfip2

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Edited by Joan S. Brugge, Harvard Medical School, Boston, MA, and approved May 20, 2010 (received for review October 9, 2009)

Ndfip1 and Ndfip2 are related endosomal membrane proteins that bind to and activate members of the Nedd4 family of E3 ubiquitin ligases. These ligases in turn affect receptor tyrosine kinase signaling by ubiquitinating several key components of the signaling pathways. Here we investigate the role of the Ndfip proteins in EGF signaling. We show that they associate with the EGF receptor and PTEN, and control the ubiquitination and abundance of PTEN, c-Cbl, and Src family kinases. Ndfip2, but not Ndfip1, also binds to and is phosphorylated by Src and Lyn, and can act as a scaffold for Src phosphorylation of Ndfip1 and potentially other substrates. Depletion of Ndfip1 inhibits Akt activation in EGF-stimulated HeLa cells, stimulates activation of Jnk, and enhances cell multiplication. Thus Ndfip1 and Ndfip2 are physically and functionally associated with multiple components of the EGF signaling cascade, and their levels modulate the relative output of different signaling pathways.

EGF | Itch | Nedd4 | Src | Cbl

Ubiquitination of proteins is an important regulatory mechanism. It targets proteins for degradation by the proteasome, and can affect the endocytosis, sorting, or lysosomal degradation of membrane proteins (1–3). It can also regulate protein activity directly or create a scaffold to which signaling molecules are recruited, most noticeably in the NFkB pathway (4). Ubiquitination involves a three-component enzyme system: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3) that dictates substrate specificity (1). Mammalian cells contain hundreds of E3 ligases, but an important subset is the Nedd4 family of ligases, of which there are nine in humans (5). These share a characteristic modular design with an amino-terminal C2 domain, two to four WW domains, and a carboxyl-terminal HECT domain with ligase activity. The C2 domain binds to phospholipids and promotes membrane association. The WW domains recognize PY motifs, such as LPXY or PPXY. Nedd4-like E3s regulate a wide variety of cellular processes, including signaling (Nedd4-1, Itch, Smurf1, Smurf2), protein trafficking (Nedd4-1, Nedd4-2), and viral budding (Nedd4-1) (5). Signaling is affected by the selective degradation of key components such as G-protein-coupled receptors, the PIP3 phosphatase PTEN, and the c-Jun and JunB transcription factors. Nedd4 members are involved in regulating the immune response (Itch, Nedd4-1), blood pressure (Nedd4-2), and bone homeostasis (Smurf1) (5). Gene amplification of one (WWP1) has been found in a substantial number of breast and prostate cancers, and it is thought that this contributes to the development of cancer by the enhanced degradation of tumor suppressors such as p53, KLF2, and KLF5 (6).

We have previously shown that the small endosomal PY-motif containing membrane proteins Ndfip1 (N4WBP5) (7) and Ndfip2 (N4WBP5A) (8) are potent activators of Nedd4 family members through multiple interactions with the WW domains (9). They also function as adaptor proteins that bring the E3s not only to endosomes but also directly to their substrates, as shown for the metal transporter DMT1 and for several substrates of the yeast Ndfip ortholog Bsd2 (10–13). The Ndfip proteins are induced upon T-cell activation, and mice that lack Ndfip1 develop severe inflammation due to T-cell hyperactivation (8, 14). This phenotype strongly resembles that of itchy mice, which lack functional Itch E3 ligase (15). Thus Ndfip1 appears to be a key regulator of the Nedd4-like Itch protein in T cells, acting to dampen signaling from the T-cell receptor.

In this paper we have examined potential roles for the Ndfip proteins in the well-characterized EGF signaling pathway common to all epithelial cells. Stimulation of the receptor tyrosine kinase (EGFR) activates a variety of pathways characterized by the downstream protein kinases that are turned on (16). Several components of these pathways have been shown to be substrates for Nedd4-like ubiquitin ligases, including Cbl, PTEN, and Jun (15, 17, 18). These proteins are thought to interact directly with the ligases, but how this might be regulated is not well understood.

Here we show that endogenous Ndfip protein levels control the ubiquitination and abundance of c-Cbl, PTEN and the Src family tyrosine kinases Src and Lyn. Ndfips physically associate with signaling components, including the EGFR and PTEN, and Ndfip2 also binds to, and is phosphorylated by, Src family kinases. Reduction of Ndfip1 levels has multiple effects, including reducing signaling through the Akt pathway, stimulating Jnk signaling, and enhancing cell growth. Thus Ndfip proteins are important components of the signaling machinery, with the ability to modulate the relative activities of different pathways emanating from a single growth factor.

Results

Ndfips Control the Levels of Signaling Components. To test the general importance of the Ndfip proteins in receptor tyrosine kinase signaling, we studied their effects on the well-characterized EGFR-dependent pathway. First, we tested siRNAs for their ability to knock down expression of myc-tagged Ndfip1 and 2 in transfected 293T cells. Figure 1*A* shows that they specifically reduced the levels of the expressed Ndfip proteins, with siRNAs 1-2 and 2-2 being particularly effective. These RNAs proved equally effective against endogenous Ndfip1 and 2 in HeLa cells, detected with appropriate antibodies (Fig. 1*B*). Lysates of siRNA-treated cells were then probed for a variety of proteins implicated in signaling. Some cells were also transfected with low levels of plasmids expressing the mouse variants of Ndfip1 and 2, which due to nucleotide sequence differences are resistant to the siRNAs. Changes due to specific effects of the siRNAs on Ndfip levels should be reversed by coexpression of the plasmids.

The findings show that several signaling proteins are very sensitive to Ndfip levels. C-Cbl and Jun, known substrates for

Author contributions: T.M. designed research; T.M. performed research; and H.R.B.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.0911714107/-/DCSupplemental.

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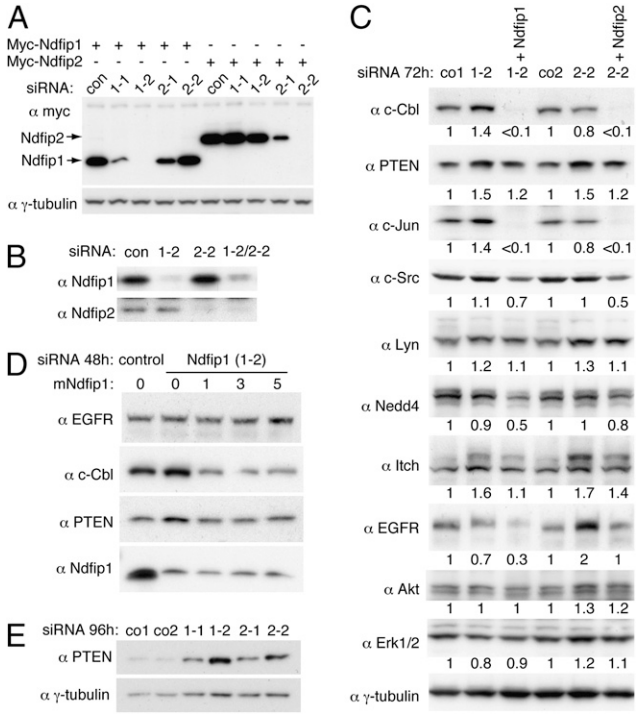

Fig. 1. SiRNA mediated knockdown of Ndfip1 and Ndfip2 changes the abundance of signaling molecules. (*A*) Blot of 293T cells transiently expressing myc-Ndfip1 or myc-Ndfip2, treated with siRNAs against Ndfip1 (1-1, 1-2), Ndfip2 (2-1, 2-2), or randomized sequences (con, co1, co2). (*B*) Immunoblots of HeLa cells transfected with the indicated siRNAs. *C* is the same as *B*, but some cells were also transfected with plasmid (50 ng/10 cm plate) expressing mouse Ndfip1 or Ndfip2, which are resistant to the siRNAs. Numbers indicate normalized protein levels. *D* is the same as *C*, but cells were harvested after 2 d. Plasmid quantities were 100 ng (1), 300 ng (3), or 500 ng (5). Note that the anti-human Ndfip1 antibody does not recognize the expressed mouse protein. *E* is the same as *C*, but cells were harvested after 4 d.

Nedd4 and Itch (15, 17), were moderately elevated by Ndfip1 knockdown, and almost eliminated by expression of either Ndfip1 or Ndfip2 (Fig. 1*C*). More modest but similar effects were seen with PTEN, Src, Lyn, and EGFR, as well as Nedd4 and Itch. The downstream protein kinases Akt and Erk, which are not known substrates for Nedd4 family ubiquitin ligases, were largely unaffected. Varying the amount of Ndfip1-expressing plasmid over a 5-fold range did not alter the levels of c-Cbl and PTEN after 48 h, indicating that the plasmid levels used, although low, were saturating for these effects (Fig. 1*D*). As a further control, we examined PTEN levels after 96 h of treatment with each of the siRNAs (Fig. 1*E*). An increase was apparent with all of the Ndfip siRNAs, although the effect was stronger with 1-2 and 2-2, correlating with their greater potency in reducing Ndfip levels (Fig. 1*A*).

The changes in protein levels are likely to be a consequence of ubiquitination and degradation, with different proteins differing in their susceptibility, or access, to Ndfip-activated ubiquitin ligases on endosomes. To test this, we focused on PTEN, whose levels are known to be a critical factor in the growth of many tumors. We expressed HA-tagged PTEN and His-tagged ubiquitin in 293T cells, with or without Ndfip proteins and E3 ligases. Following Ni-NTA (nickel-nitrilotriacetic acid) purification, ubiquitinated PTEN was detected by immunoblotting. Figure 2*A* shows that expression of either Ndfip1 or Ndfip2 could induce poly-ubiquitination of PTEN by either Nedd4 or Itch. Notably, in their absence, Nedd4 and Itch were almost completely inactive.

Immunoprecipitation of Ndfip1 and 2 from a similar experiment showed that at least some of the PTEN was associated with them. Although Nedd4 has been shown to bind PTEN directly (18), the association of PTEN with Ndfip1 and 2 was not increased by coexpression of Nedd4, nor decreased by mutation of the PY elements in the Ndfip proteins, which greatly reduces their interaction with Nedd4 ligases (Fig. 2*B*). Thus, ubiquitination of PTEN may be facilitated by an additional interaction, direct or indirect, with Ndfip proteins.

We could also detect increased ubiquitination of endogenous PTEN when Nedd4 was coexpressed in 293T cells with Ndfip2, but not when it was expressed with a version of Ndfip2 in which the three PY motifs were mutated (Fig. 2*C*). Ubiquitination of endogenous Src and c-Cbl was also increased under these conditions. Finally, we could show that modification of endogenous PTEN and Src was increased when either Nedd4 or Ndfip1 was expressed alone, although less so than when both were expressed together (Fig. 2*D*). Thus, the ubiquitination and hence degradation of these proteins is regulated in normal cells by the levels not only of Nedd4 but also of the Ndfip proteins.

EGFR-Dependent Tyrosine Phosphorylation of Ndfips. Because Ndfip levels affect several signaling components that are known to interact, notably c-Cbl, Src, and the EGFR, we investigated whether the Ndfips, which are found in endosomes, are physically associated with signaling complexes. Figure 3*A* shows that Ndfip2 expressed in HeLa cells colocalized with some of the endogenous EGFR in intracellular dot-like structures, most likely representing the endosomal compartments in which the Ndfips have previously been detected (19). Identical results were obtained with Ndfip1 (Fig. S1). This suggests that the EGFR meets the Ndfip proteins following its endocytosis, which rapidly follows EGF binding (20).

Further evidence that the Ndfips are closely associated with signaling came from the observation that EGF stimulation of serum-starved 293T cells resulted in a transient tyrosine phosphorylation of expressed Ndfip1 and Ndfip2 (Fig. 3*B*). This phosphorylation was reduced when an inhibitor of Src family kinases (PP2) was added, suggesting that phosphorylation was mediated not by the EGFR itself, but by Src molecules that are activated by the EGFR. Further investigation confirmed that Ndfip2 was readily phosphorylated by activated Src in vitro (Fig. S2).

Because c-Cbl and Src physically associate with the EGFR, and Ndfip2 is affected by, and affects, all three proteins, we sought direct evidence for an association of Ndfips with the EGFR by coexpressing these proteins. Under these conditions, the relatively high levels of EGFR lead to activation of its tyrosine kinase activity and autophosphorylation. Figure 3*C* shows that this also resulted in phosphorylation of Ndfip1 and Ndfip2. Moreover, phosphorylated EGFR coprecipitated with them.

To examine this interaction under more natural conditions, we attempted to precipitate endogenous Ndfip proteins. However, our antibodies proved inadequate for this. Instead, we prepared a stable line of A431 cells that expressed myc-tagged Ndfip2, at a level lower than that of the endogenous protein and hence unlikely to perturb the cells (Fig. 3*D*). Phosphorylation of this myc-Ndfip2 was low under normal growth conditions. Addition of EGF to serum-starved cells resulted in increased phosphorylation of both Ndfip2 and EGFR. Importantly, coimmunoprecipitation of EGFR with Ndfip2 increased following EGF stimulation, peaking at the 10-min time point (Fig. 3*E*). Thus, Ndfip2 is physically and functionally connected to the EGF signaling machinery shortly after EGF stimulation, presumably in endosomes after internalization of the activated receptor.

Phosphorylation of Ndfip2 Provides an Additional Binding Site for Src Family Kinases. Coexpression of Ndfip2 tyrosine mutants with a series of activated Src family kinases (Src, Fyn, Lyn) revealed

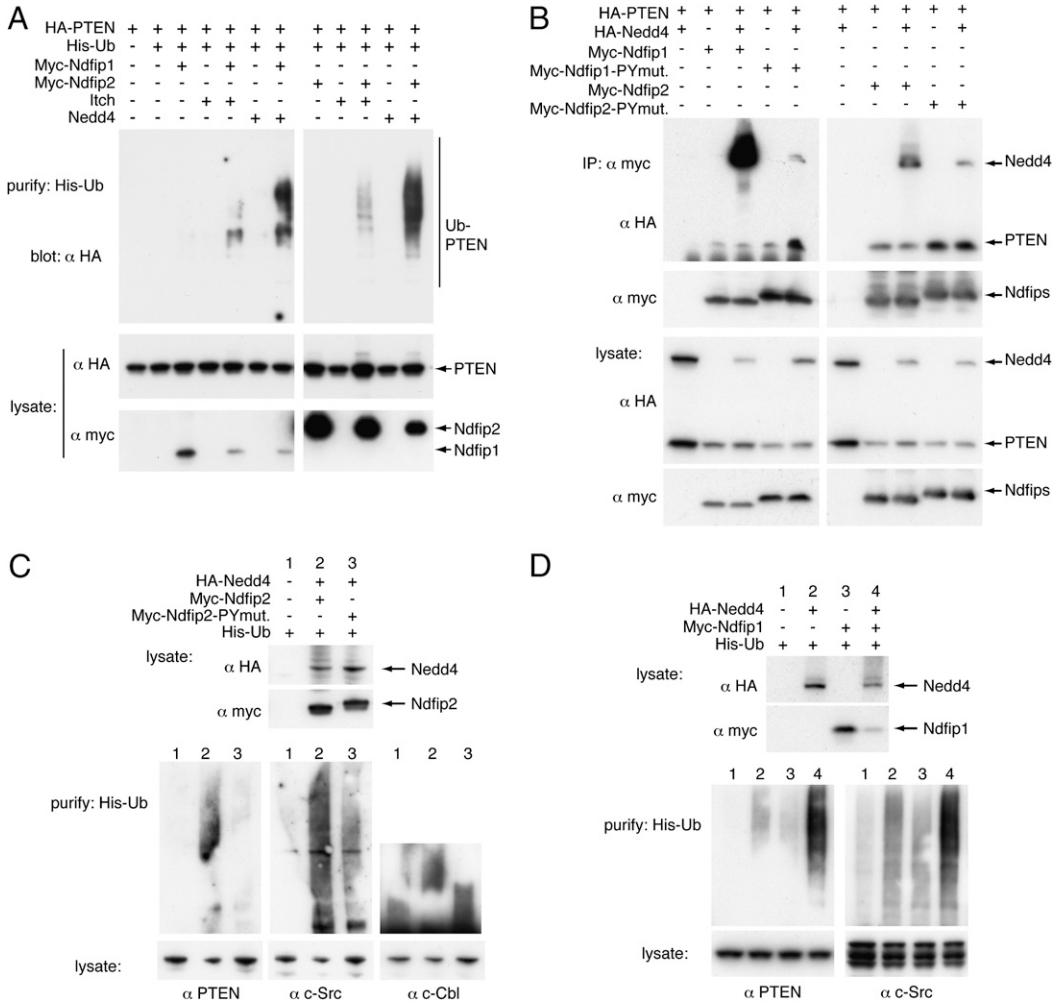

Fig. 2. Ndfip proteins promote ubiquitination of PTEN and other proteins by Nedd4-like E3 ligases. (*A*) 293T cells were cotransfected with plasmids expressing His-tagged Ubiquitin, HA-tagged PTEN, myc-tagged Ndfip proteins, and Itch or Nedd4 as indicated. Ubiquitinated proteins were isolated using Ni-NTA agarose, and these and whole-cell lysates analyzed by immunoblotting. Exposures of the two upper panels were adjusted to compensate for the different expression levels of Ndfip1 and Ndfip2 in this experiment. (*B*) PTEN coprecipitates with Ndfips. 293T cells were transfected and analyzed as indicated. PYmut indicates Ndfip versions in which each PY motif was mutated from PxY to PAG. (*C* and *D*) 293T cells were transfected as indicated and immunoblotted for the expressed proteins and also endogenous PTEN, c-Src and c-Cbl. Ubiquitinated forms appear as smears. Note that c-Cbl binds to Ni-NTA; this unmodified protein has been cut off the blot. Cbl autoubiquitinates, but in lane 2 the Ub chains are longer indicating enhanced modification.

that Y73 was critical for phosphorylation (Fig. 4*A*). Additional experiments showed that Y57, Y77, and Y83 were also phosphorylated, but that modification of these tyrosines is absolutely dependent on Y73 (Fig. S3).

Src and Lyn could also be coprecipitated with Ndfip2 (Fig. 4*B*). Under similar conditions, Ndfip1 was more weakly phosphorylated than Ndfip2 and interacted with the kinases, particularly Lyn, much less well. Lyn association with Ndfip2 was primarily dependent on its SH3 domain (Fig. 4*C*). However, because Y73 seemed important for further modification of Ndfip2, we considered the possibility that phosphorylation of this residue aids interaction with the kinases by providing an additional binding site for their SH2 domains (21). As a direct test of this we phosphorylated the Ndfip2 cytoplasmic domain in vitro and bound it to GST fusion proteins containing either the SH3 domain, or both the SH3 and SH2 domains of Lyn. SH2-dependent binding to phosphorylated Ndfip2 was indeed observed (Fig. 4*C*).

Although Ndfip1 bound Src family kinases poorly, it was phosphorylated following EGF stimulation (Fig. 3*B*). However, we also observed that Ndfips are capable of associating with each other (Fig. S4). Ndfip1 may thus be phosphorylated mainly because it associates with Ndfip2, which in turn recruits Src. In agreement with this, we found that though coexpression of Ndfip1 and active Src produced only a very low level of Ndfip1 phosphorylation, additional expression of Ndfip2 greatly enhanced this (Fig. 4*D*). The enhancement activity of Ndfip2 specifically required Y73. Thus at least under these conditions, phosphorylation of Y73 allows Ndfip2 to act as a scaffold that recruits Src kinase to an associated protein, namely Ndfip1. Phosphorylation of Ndfip1 occurred mainly on the tyrosine residues of the PY elements, as judged by the loss of signal when these were mutated (Fig. S5).

Because we had already shown that Ndfip2 can enhance ubiquitination of Src kinases, we wondered whether its phosphorylation would also contribute to this process by increasing the probability of simultaneous recruitment of kinase and ubiquitin ligase. To test this directly, we used an in vitro ubiquitination assay. This assay uses relatively dilute soluble components, and consequently is very sensitive to the strength of direct protein-protein interactions. We found that ubiquitination of an inactive version of Lyn (lacking the kinase domain) by Itch was stimulated more effectively by a phosphorylated form of the Ndfip2 cytoplasmic domain than by the unphosphorylated form (Fig. 5*A*).

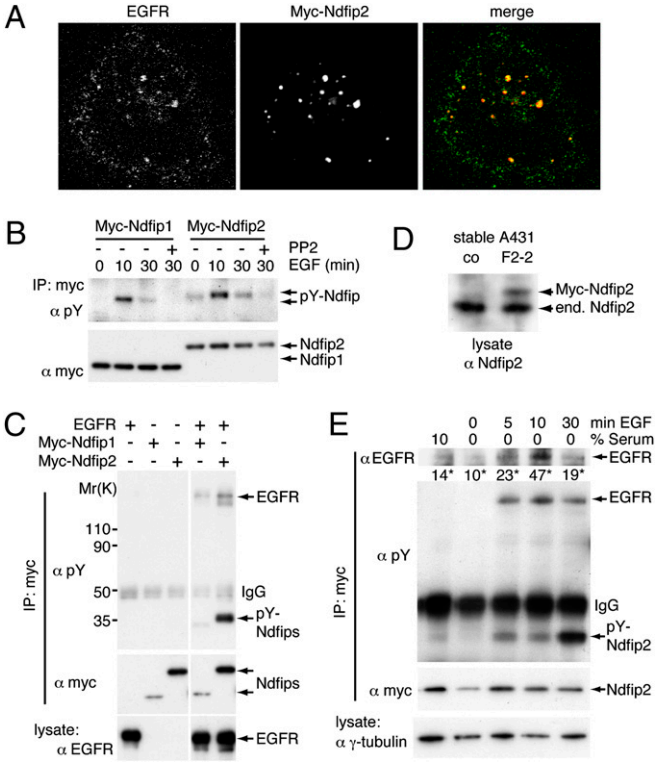

Fig. 3. EGFR-dependent tyrosine phosphorylation of Ndfips. (*A*) Immunofluorescence of endogenous EGFR and transiently expressed myc-Ndfip2 in HeLa cells. (*B*) Phosphorylation of Ndfips. 293T cells expressing myc-tagged Ndfips were starved overnight, then treated with 100 ng/mL EGF for 10 or 30 min. PP2 (10 μM) was added 10 min before EGF as indicated. (*C*) Coprecipitation of Ndfip proteins and EGFR expressed in 293T cells. (*D*) Control A431 cells (co) and A431 cells stably expressing myc-Ndfip2 (F2-2) were probed for the relative expression levels of endogenous and exogenous Ndfip2 using an Ndfip2 antibody. (*E*) Coprecipitation of endogenous EGFR with Ndfip2. A431 cells stably expressing Myc-Ndfip2 were grown in 10% serum, or serum starved overnight before addition of EGF (100 ng/mL) for the times indicated. Myc-Ndfip2 was immunoprecipitated, and samples analyzed by immunoblotting. Numbers (*) are amounts of coprecipitating EGFR, normalized to Ndfip2, in arbitrary units. Note that signaling persists in A431 cells under these conditions, causing accumulation of phospho-Ndfip2.

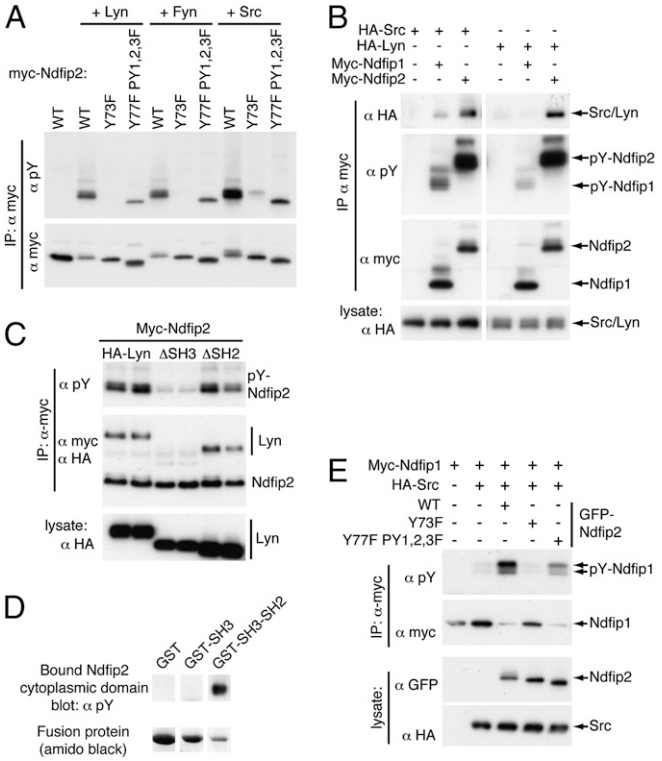

Fig. 4. Interaction of Src family kinases with Ndfip2. (*A*) Phosphorylation requires Y73. Myc-Ndfip2 or tyrosine mutant versions were coexpressed in 293T cells with constitutively active versions of Lyn, Fyn, or Src. (*B*) Kinases bind Ndfip2. Ndfip1 and Ndfip2 were coexpressed with Src and Lyn and immunoprecipitated. (*C*) The Ndfip2-Lyn interaction is SH3 domain dependent. Ndfip2 was immunoprecipitated from cells coexpressing constitutively active intact Lyn or SH3 or SH2 deletions as indicated. (*D*) The SH2 domain interacts with phospho-Ndfip2. Recombinant Ndfip2 cytoplasmic domain was phosphorylated in vitro with purified Src, then bound to GST constructs bearing the Lyn SH3 and SH2 domains. (*E*) Y73 is required for Ndfip2 to mediate Src phosphorylation of Ndfip1. Ndfip1 was coexpressed in 293T cells with active Src and WT GFP-Ndfip2 or different tyrosine mutants.

Versions of Ndfip2 with mutated PY motifs lacked activity, as expected.

In vivo, the effect was more modest. Expression of active Lyn resulted in its ubiquitination, and this was enhanced by coexpression of Ndfip2 (Fig. 5*B*). The effect was reduced when the PY elements were mutated, and somewhat reduced when Y73 was absent. Most strikingly, however, Lyn ubiquitination was completely dependent on its SH3 domain (Fig. 5*B*). In agreement with this, coprecipitation of Lyn with Ndfip2 did not require Y73, or the PY elements of Ndfip2 (Fig. S6). Thus, although phospho-Y73 does provide a binding site for the SH2 domain of Lyn, it is not crucial for ubiquitination in vivo.

Differential Effects of Ndfip Proteins on EGFR Signaling. Given that the Ndfips are closely associated with EGF signaling components and influence their ubiquitination and abundance, one would expect the levels of Ndfip proteins to influence downstream signaling events. To test this, HeLa cells were treated with siRNA against Ndfip1 or Ndfip2. After 48 h, a time at which EGFR levels were not significantly affected, the cells were transiently stimulated with EGF, and activation of key kinases was tested by immunoblotting with appropriate phospho-specific antibodies (Fig. 6 *A*–*C*). The EGFR activates both Ras and PI3K pathways (summarized in Fig. 6*D*) (16, 22). Ras triggers kinase cascades that activate Erk and, in a separate branch of the pathway, Jnk. PI3K produces PI3P on the plasma membrane, which if not destroyed by the PTEN lipid phosphatase recruits PDK1 and Akt, with consequent activation of Akt. We tested these kinases under normal conditions, and also when cells were treated with U0126, a specific inhibitor of MEK1 and MEK2 that inhibits Erk signaling and improves the response of the Akt and Jnk pathways.

Figure 6*A* shows that knockdown of Ndfip1 significantly reduced Akt activation. This reduction is the consequence of PTEN stabilization, because simultaneous knockdown of PTEN itself reversed the effect of Ndfip1 depletion (Fig. 6*B*). Expression of siRNA-resistant (mouse) Ndfip1 also reversed the siRNA effect, confirming specificity (Fig. 6*C*). Ndfip depletion also enhanced early activation of the Jnk pathway (Fig. 6*A*). This may be due to reduced Itch-dependent inactivation of MKK4, the kinase that activates Jnk (23). In general, knockdown of Ndfip2 had less of an effect, perhaps because it is expressed at lower levels than Ndfip1 in these cells. However, it did lead to a small but reproducible prolongation of Erk activation.

These findings show that the Ndfip proteins differentially modulate EGFR signaling through multiple pathways. In particular, this suggests that endogenous levels of Ndfip1 regulate the ratio of Akt-to-Jnk signaling in response to EGF.

We also examined the effects of Ndfip knockdown on cell growth. Ndfip2 knockdown had rather modest and variable

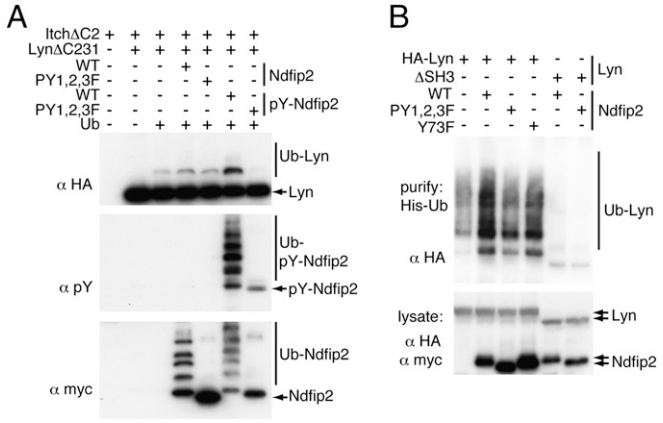

Fig. 5. Ndfip2 promotes ubiquitination of active Lyn. (*A*) In vitro ubiquitination assay using recombinant Itch lacking the membrane-binding C2 domain (ItchΔC2) as E3, recombinant Lyn protein without kinase domain (LynΔC231), and wild-type or PY mutant (PxY to PxF) cytoplasmic domains of Ndfip2 that were either nonphosphorylated or in vitro tyrosine phosphorylated by recombinant Src as indicated (pY-Ndfip2). Ubiquitination assays were analyzed by immunoblotting. (*B*) 293T cells were cotransfected with plasmids expressing His-tagged Ubiquitin and mutant or wild-type versions of Ndfip2 and Lyn. Ubiquitinated proteins were isolated using Ni-NTA agarose and probed with anti-HA to detect Lyn.

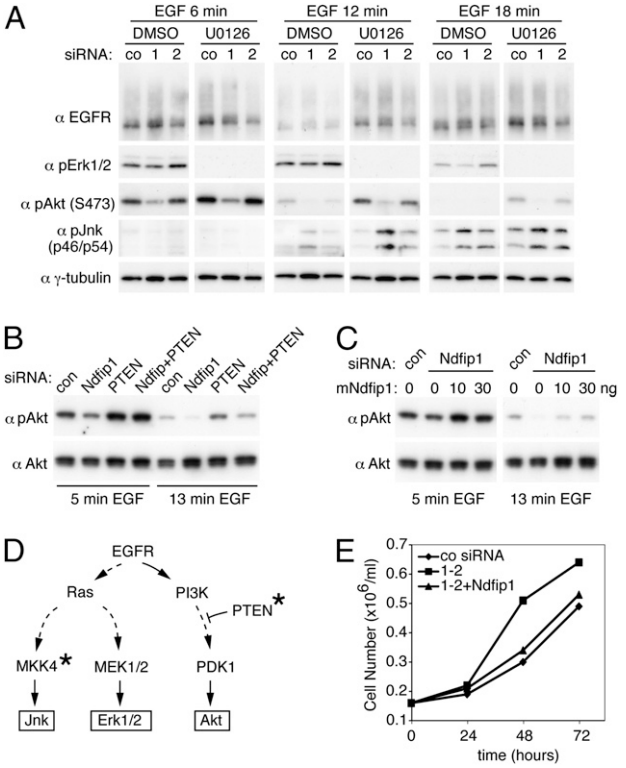

Fig. 6. Ndfip proteins differentially affect signaling pathways. (*A*) Immunoblots of HeLa cells transfected with siRNAs against Ndfip1 (1) or Ndfip2 (2) or a control siRNA (co), stimulated with 100 ng/mL EGF for the times indicated in the presence of solvent (DMSO) or the Erk inhibitor U0126 (10 μM). Activation levels of Erk1/2, Akt, and Jnk were detected using phospho-specific antibodies (pErk1/2, pAkt, pJnk). (*B*) PTEN siRNA reverses the effect of Ndfip1 siRNA on Akt activation. (*C*) Expression of siRNA-resistant (mouse) Ndfip1 reverses the effect of Ndfip1 siRNA on Akt activation. (*D*) Summary of the major EGF-induced signaling pathways. Known substrates for Itch ubiquitin ligase are marked by an asterisk. (*E*) Growth of HeLa cells following a single transfection with a control siRNA (co) or one targeted to Ndfip1 (1 and 2), with or without a plasmid expressing siRNA-resistant (mouse) Ndfip1. Plasmid levels were titrated to a level that did not induce cell death, which was noticeable at higher levels.

effects. Strikingly, however, reducing Ndfip1 levels with siRNA consistently increased the rate of HeLa cell growth, and this effect was specific because it could be reversed by coexpressing mouse Ndfip1 (Fig. 6*C*). This was surprising, because Akt signaling, which is often associated with cell growth, is reduced under these conditions. However, Ndfip1 affects the levels of many different proteins, and the physiological outcome is not easy to predict. Nevertheless, we can conclude that Ndfip1 is an important variable in these processes.

Discussion

Ndfip proteins are potent recruiters and regulators of, and adaptors for, Nedd4 family ubiquitin ligases on endosomal membranes (9). Here we show that the endogenous levels of these proteins in tissue culture cells significantly modulate the pathways of EGFR signaling. Notably, they control PTEN ubiquitination and hence its abundance, with critical consequences for signaling through Akt kinase. They also influence Jnk signaling, most likely through the ubiquitination of upstream activators. In addition, they regulate the ubiquitination and abundance of c-Cbl and of Src family kinases, have a modest effect on the kinetics of signaling through the Erk pathway, and influence cell growth.

These properties are likely to be of physiological importance. Both Ndfip1 and Ndfip2 are transcriptionally up-regulated following mitogenic stimulation of T lymphocytes (8). Ndfip ($^{-/-}$) T cells are hyperactivated, a phenomenon that has been ascribed to a deficiency in Itch-mediated degradation of Jun transcription factors (14). Our findings suggest that a second effect is the increased activation of Jnk and hence Jun. This may be due to a failure of Itch to inactivate the upstream kinase MKK4 (23), although because Itch also interacts with MEKK1 (24), which phosphorylates MKK4, and Akt can also indirectly affect Jnk signaling (25), there may be multiple effects. Jnk also phosphorylates and activates Itch, thus creating a feedback loop that should inhibit Jnk activation (26, 27), but this is evidently insufficient to regulate Jnk in the absence of Ndfip1.

The importance of the PTEN-Akt pathway is well illustrated by its very frequent activation in a wide variety of tumors. For example, a high proportion of breast cancers show loss of PTEN, activation of Akt, or mutation or increased copy number of PI3K (28). Our findings indicate that overexpression of Ndfip proteins should have a similar effect to loss of PTEN, and indeed Ndfip1 mRNA (under the name SGA-1M) was shown to be overexpressed in 40% of breast tumors (29). However, in HeLa cells we found that growth was stimulated by a reduction in Ndfip1 levels, and observed that overexpression at high levels led to cell death. This underscores the fact that Ndfips, as general stimulators of Nedd4 family ligases, can have many different effects. It is likely that the tumors that overexpress Ndfip1 have acquired other changes that allow them to avoid its negative effects on growth.

Src Family Kinases. An unexpected finding was that Ndfip2 has a specific affinity for Src family kinases, and is a substrate for them. This indicates a functional difference between Ndfip1 and Ndfip2, and that their relative levels may be important for the fine-tuning of signaling pathways.

Src is activated upon phosphorylation by ligand-bound EGFR (30). Because Ndfip2 is closely associated with the EGFR following EGF stimulation, it will be well placed to interact with Src. This leads to phosphorylation of Y73, which may further increase the affinity for Src by providing an additional binding site for the SH2 domain. The association of active Src with Ndfip2 can then have several consequences.

The most obvious one is that Src is presented to Nedd4 family ubiquitin ligases, and we have demonstrated in vitro and in vivo

that ubiquitination of Src and Lyn occurs. Interestingly, increasing the level of Ndfip1 also stimulated ubiquitination and degradation of these kinases, even though Ndfip1 does not bind them efficiently. This may simply be because Ndfip1 recruits ubiquitin ligases to a signaling complex that also contains Src kinases anchored to other proteins, including Ndfip2.

A second potential consequence that we have not investigated is the presentation of Nedd4 ligases to Src family kinases. It has been reported that the Fyn can tyrosine phosphorylate Itch in T cells, and that this inhibits Itch-mediated ubiquitination of JunB (31). Itch phosphorylation was reported to require the third WW domain of Itch and the SH3 domain of Fyn; it seems possible that the two proteins meet on the scaffold provided by Ndfip2—meaning that Ndfip2 would both activate Itch and facilitate its inactivation.

More broadly, by recruiting kinases, Ndfip2 can present them to other proteins with which it interacts. We have demonstrated that one substrate that can be phosphorylated in this manner is Ndfip1. This role seems to require the increased affinity provided by Y73 phosphorylation, and in general Ndfip2 may serve to prolong the association of active Src kinases with the signaling complex. It remains to be determined whether it also influences the phosphorylation of other known Src substrates with which it associates, such as PTEN (32), the EGFR itself (30), or perhaps Cbl, which ubiquitinates the EGFR (33, 34).

In conclusion, we have shown that Ndfip proteins are physically and functionally associated with multiple components of the EGF signaling cascade. Moreover, decreasing or increasing their abundance has readily detectable consequences, including altering the relative strength of signaling through different pathways. Their levels are thus important variables in the modulation of these pathways.

Materials and Methods

Plasmids. All ORFs were cloned into pcDNA3.1 (Invitrogen) without tag (EGFR), with a single amino-terminal myc-tag (Ndfip1 and Ndfip2) (9) or a triple N-terminal hemagglutinin (HA) tag (PTEN, Src, Lyn). In addition, carboxyl-terminal tagged versions of Src and Lyn with mCherry or Fyn with mRFP (Clontech) were used. For recombinant protein expression, all constructs were inserted into either pGEX6P-2 (GE Healthcare) or pET28a (Novagen). All kinase constructs were constitutively active point mutants: Src (Y527F), Lyn (Y508F), Fyn (Y531F). Mutations were introduced using the QuikChange Kit (Stratagene). Further details are provided in *SI Materials and Methods*.

Other Techniques. Transfection of HEK293T or HeLa cells, immunoprecipitation, purification of ubiquitinated and recombinant proteins, immunoblotting, and in vitro ubiquitination assays were performed essentially as described previously (9). Antibodies were from Abcam (Itch, Nedd4), Cell Signaling Technology/NEB (PTEN, pS473Akt, Akt, pErk1/2), Upstate (pY 4G10), Santa Cruz (EGFR, Src), or Sigma (Ndfip1, Ndfip2, γ-tubulin, myc, HA). EGF was from Invitrogen, PP2 from Calbiochem, and U0126 from Cell Signaling Technology. Immunofluorescence and siRNA transfections were by standard procedures. Details are given in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Elina Nikko and Mike Lewis for helpful discussions, Kirsi Riento (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK) for the Src family kinase-fluorescent protein constructs, and Mariann Bienz and Ben Nichols for comments on the manuscript.

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