Sprouty (Spry) proteins are important regulators of receptor tyrosine kinase signaling in development and disease. Alterations in cellular Spry content have been associated with certain forms of cancers and also in cardiovascular diseases. Thus, understanding the mechanisms that regulate cellular Spry levels are important. Herein, we demonstrate that Spry1 and Spry2, but not Spry3 or Spry4, associate with the HECT domain family E3 ubiquitin ligase, Nedd4. The Spry2/Nedd4 association involves the WW domains of Nedd4 and requires phosphorylation of the Mnk2 kinase sites, Ser112 and Ser121, on Spry2. The phospho-Ser112/121 region on Spry2 that binds WW domains of Nedd4 is a novel non-canonical WW domain binding region that does not contain Pro residues after phospho-Ser. Endogenous and overexpressed Nedd4 polyubiquitinate Spry2 via Lys18 on ubiquitin and decrease its stability. Silencing of endogenous Nedd4 increased the cellular Spry2 content and attenuated fibroblast growth factor factor-elicted ERK1/2 activation that was reversed when elevations in Spry2 levels were prevented by Spry2-specific small interfering RNA. Mnk2 silencing decreased Spry2-Nedd4 interactions and also augmented the ability of Spry2 to inhibit fibroblast growth factor signaling. This is the first report demonstrating the regulation of cellular Spry content and its ability to modulate receptor tyrosine kinase signaling by a HECT domain-containing E3 ubiquitin ligase.

Mammalian Sprouty (Spry) proteins have emerged as important modulators of the biological actions of receptor tyrosine kinases (RTKs). The four Spry isoforms (Spry1–4) are products of different genes located on different chromosomes (1). All four isoforms retain significant similarity to the first member of this family that was discovered in Drosophila (2). The C-terminal half of all Spry isoforms is Cys-rich, and retains the highest degree of similarity among them (reviewed in Ref. 3). Although the N-terminal half of the Spry proteins is more variable, some features in this region are retained in all isoforms (4). For instance, all Spry isoforms have a conserved Tyr residue in the N terminus that is phosphorylated by growth factors and serves as a docking site for the Src homology 2-like tyrosine kinase-binding domain of c-Cbl (5–9). However, c-Cbl does not associate with each Spry isoform equally; interaction between c-Cbl and Spry2 appears to be stronger than that with Spry4 (10). Because of their differential expression in various tissues and the variability in their N terminus, the precise mechanism of action of each Spry isoform may vary (4).

Because Spry proteins inhibit the biological actions of several growth factors on tubular morphogenesis including angiogenesis (reviewed in Refs. 4 and 11–13) as well as cell migration and proliferation (14–20), they were referred to as inhibitors of RTKs. However, Spry proteins can also positively regulate the biological actions of RTKs and their signaling. Thus, by binding c-Cbl and sequestering it away from growth factor receptors such as the EGF receptor, Spry2 augments extracellular signal-regulated kinase (ERK) activation by EGF and induces neurite outgrowth in PC12 cells (7, 21, 22). Likewise, by sequestering c-Cbl, Spry2 also facilitates the anti-apoptotic actions of serum (23).

Consistent with its role as a negative regulator of RTK signaling, Spry2 content in several forms of tumors including breast, prostate, hepatocellular, and non-small cell lung carcinomas is decreased (24–27). Likewise, in cardiac hypertrophy and in the failing heart, elevated levels of microRNA 21 decrease the levels of Spry2 and Spry1 in cardiac myocytes and fibroblasts, respectively, and enhance ERK1/2 signaling, morphological changes, and cardiac remodeling (28, 29). In contrast, the role of Spry2 as positive modulators of RTKs may be pertinent in regulating the growth and metastasis of oncogenic H-Ras-transformed cells (30) and melanomas (31) where its cellular level is elevated. Moreover, the elevated Spry2 content by protecting the mutant, activated form of fibroblast growth factor receptor 3 from down-regulation, exacerbates the negative influence of FGF receptor 3 on proliferation and terminal differentiation of chondrocytes in growth plates in type II thanatophoric dysplasia (32).

Clearly, cellular Spry content can contribute toward, or exacerbate, certain diseases. Hence, an understanding of the mechanisms that regulate Spry expression as well as degradation is important. Spry2 is degraded mainly via the proteosomal pathway, and to a lesser extent by the lysosomal pathway (7, 8). To date, two ring-finger E3 ubiquitin ligases, c-Cbl and overexpressed Siah2, have been demonstrated to regulate Spry2 levels (7, 8, 22, 33). The phosphorylation of Tyr55 in the N terminus of Spry2 provides a docking site for c-Cbl, which as described...
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above can augment signaling via RTKs. Additionally, by providing a binding site for c-Cbl, Spry2 permits its own ubiquitination by the E3 ligase (6, 7, 21). The N terminus of Spry2 also interacts with the ring finger domain of Siah2 and, unlike c-Cbl, tyrosine phosphorylation of Spry2 is not necessary for this interaction (33). Overexpression of Siah2 also results in proteosomal degradation of Spry2, Spry1, and Spry4 with an increase in FGF-mediated ERK1/2 activation (33). Notably, however, whether Siah2 ubiquinates Spry2 or a role of endogenous Siah2 in regulating Spry2 levels remains unknown.

In this report, we demonstrate that Nedd4, a HECT domain family E3 ubiquitin ligase, binds to and ubiquinitates Spry2. The interaction between these proteins involves the WW domains of Nedd4 and a region encompassing Ser112 and Ser121 on Spry2. Phosphorylation of Ser112 and Ser121, Mnk2 sites, increases interaction between Spry2 and Nedd4. Silencing of endogenous Nedd4 decreased the ubiquitination of Spry2 and increased its stability. Moreover, silencing of Nedd4 increased Spry2 content and decreased FGF-mediated activation of ERK1/2. This is the first demonstration of a HECT domain-containing E3 ubiquitin ligase in the regulation of Spry2 ubiquitination and stability.

MATERIALS AND METHODS

Plasmids and Constructs—The cloning of the human Spry2 cDNA in the pHM6-HA vector has been described (14). The various Spry2 mutants were generated using universal PCR with mutagenic primers. FLAG-tagged Spry2 was constructed after double digestion of pHM6-HA-SPRY2 with KpnI and EcoRI restriction enzymes and reverse-cloning the Spry2 insert into pXJ40-FLAG vector using KpnI/EcoRI restriction sites. Spry1, Spry3, and Spry4 cDNAs in the pXJ40-FLAG vector were generous gifts from Dr. Graeme R. Guy, Institute of Molecular and Cell Biology, Singapore. T7-tagged WT rat Nedd4 (rNedd4), catalytically inactive C867S rat Nedd4, pCMV-3X-FLAG-ubiquitin, FLAG-AIP4, and GST-AIP4 WW1–4 constructs were kindly provided by Dr. Adriano Marchese, Loyola University Chicago. V5-tagged human NEDD4-1 (hNEDD4-1) was a gift from Dr. Daniela Rotin, University of Toronto. HA-tagged WT, K48R, and K63R ubiquitin constructs were provided by Dr. Joanna Bakowska, Loyola University Chicago. All constructs were verified and confirmed by sequencing.

GST-WW Domain Fusion Proteins—Using full-length rNEDD4 and hNEDD4-1 as templates, cDNAs corresponding to WW domains 1–3, 1–2, and 3 of rNedd4 and WW domains 1–4, 1–2, and 3–4 of hNEDD4-1 were generated by PCR and cloned in pGE4-4T-3 vector (GE Healthcare). The constructs in pGEX-4T-3 were transformed into the BL21(DE3) strain of Escherichia coli (Promega), and single colonies were grown overnight in 5 ml of Luria broth (LB) containing ampicillin. Aliquots of these cultures were transferred to 25 ml of LB containing ampicillin in 50-ml culture tubes and grown until A600nm of 0.4 and then induced with 0.1 mM isopropyl β-D-thiogalactopyranoside to express the proteins at 18 °C for 1 h followed by centrifugation at 2,500 × g for 7 min at 4 °C. Cell pellets were dissolved in 1 ml of lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol and a mixture of protease inhibitors) and sonicated for 10 s on ice before centrifugation at 20,000 × g for 20 min. The clear supernatant was mixed with 100 µl of glutathione-Sepharose™ 4B beads (GE Healthcare) and incubated for 1 h at 4 °C. Bound proteins were pelleted (4,000 × g, 5 min) and the beads were washed three times before re-suspending in a 150-µl final volume. Aliquots were checked for purity and concentration by SDS-PAGE.

Silencing of Nedd4-1, Spry2, Mnk1, and Mnk2—HEK293T cells (1 × 106 cells/10-cm dish) were transfected with 1 µg each of the various Nedd4 shRNA (V2LHS_254872, V2LHS_72555, and V2LHS_72553) or control shRNA constructs obtained from Open Biosystems (Huntsville, AL). After 48 h, cells were treated with 2 µg each of puromycin in 1 ml of culture medium. Puromycin-resistant stable Nedd4 shRNA expressing polyclonal cells were selected and maintained in 1 µg/ml of puromycin. Cells in the early passages were used for further experiments. To silence Spry2 expression in HeLa cells, moderately overexpressing Spry2 (Clone3 (14)), 27-mer Spry2 siRNA, or mutant siRNA harboring 3 ribonucleotide substitutions were used as described previously (23). Mnk1 was silenced using a 25-mer stealth siRNA duplex from Invitrogen (catalog number 10620318/9) with the following sequence: sense 5′-CCU UGC CAG GAA AGU UUG AAG AUA U-3′, antisense 5′-AUA UCU UAC UUU CCC AAG G-3′. Mnk2 was silenced using a 25-mer stealth siRNA duplex (Invitrogen; catalog number VHS40576) with the following sequence: sense 5′-CUG AGU AGG AUA UAC AAG AGC C-3′, antisense 5′-GCC UCU UGU AUA UCC UAC UCA G-3′.

Immunoprecipitations (IPs) and Western Blotting—For co-IP and pulldown experiments HA- or FLAG-tagged Spry constructs were transfected with or without C867S T7-rNedd4 in HEK293T cells (750,000 cells/60-mm dish). After 48 h, cells were placed on ice, media was removed, washed twice with ice-cold PBS before lysing in 400 µl of lysis buffer as described (18). About 500 µg of cell lysate was used to IP Spry proteins using 1 µg each of the relevant antibody for 2 h at 4 °C. IPs were washed three times with lysis buffer before eluting the proteins with 30 µl of reducing Laemmli sample medium and boiling at 95 °C for 5 min. Eluted proteins were separated by 10% SDS-PAGE. For GST pulldowns, 500 µg of cell lysates were incubated with 2 µg of GST-WW domain proteins (~10 + 20 µl of Protein G beads) at 4 °C for 2 h. GST alone was used as negative control. Proteins bound to beads were washed and eluted with reducing Laemmli sample medium and separated on 10% gels. For Western analyses, membranes were blocked either in Tris-buffered saline containing 5% (w/v) nonfat dry milk with 0.1% Tween 20 or in Tris-buffered saline containing 5% (w/v) nonfat dry milk. The following antibodies were used: HA-horseradish peroxidase (3F10, Roche), HA (monoclonal, Covance), T7 (Novagen, Madison, WI), Spry2 (both N-terminal and C-terminal specific antibodies, Sigma), FLAG (M2, Monoclonal), Mnk2 (Sigma), Nedd4 and ERK1/2 (Millipore, CA), Actin (MP Biomedicals, LLC, Ohio), pERK1/2 and Mnk1 (Cell Signaling), and c-Cbl (BD Biosciences).

Ubiquitination Assays—HEK293T cells (750,000/60-mm dish) were transfected with 1 µg each of the HA-tagged Spry2 constructs, 2 µg of FLAG-tagged ubiquitin, and 1 µg each of the Nedd4 constructs. After 48 h, cells were treated with MG132 (25 µM) and incubated for 4 h at 37 °C. The cells were then
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Sprouty2 Interacts and Co-localizes with Nedd4—We first investigated whether the endogenous HECT family ubiquitin ligase, Nedd4 interacts with endogenous Spry2. As shown in Fig. 1A, endogenous Nedd4 was co-immunoprecipitated with endogenous Spry2 using a C terminus antibody against Spry2. Moreover, the Nedd4/Spry2 interaction was constitutive and not altered by treatment of cells with epidermal growth factor or serum

RESULTS

Previously, we showed that in certain cell types such as HeLa, Spry2 increases the amount of phosphatase and tensin homolog on chromosome 10 (PTEN) (34). Recent reports demonstrated that in HeLa cells PTEN is polyubiquitinated and targeted for degradation by the E3 ubiquitin ligase Nedd4 (35, 36). Hence, we postulated that akin to the interactions between c-Cbl and

Spry2 Stability Studies—HEK293T cells or Nedd4 shRNA expressing stable HEK293T cells (300,000/35-mm dish) were transfected with the indicated plasmids or their respective controls. After 48 h, cells were fixed as described under “Materials and Methods” and used for immunofluorescence analyses as described. Images shown are ×400 magnification and the portion in the box is shown at ×3 greater magnification.

Immunofluorescence—HEK293T cells were transfected with empty plasmid or WT HA-Spry2. Forty-eight hours after transfection, cells were fixed with 4% formaldehyde in 1× PBS for 15 min at room temperature. After permeabilizing the cells with 0.3% Triton X-100 in PBS for 10 min, cells were blocked with 10% goat serum in PBS for 1 h. Rabbit anti-Nedd4 (1:250 dilution) and monoclonal anti-HA (1:250 dilution) antibodies were added and incubated overnight at 4 °C. The secondary antibodies were goat anti-rabbit conjugated with Alexa Fluor 594 or goat anti-mouse conjugated to Alexa Fluor 488 (1:500 dilution). Confocal images were obtained using a multiphoton Zeiss LSM 510 laser scanning confocal microscope.

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Regulation of Sprouty2 by Nedd4

Nedd4 have been reported to interact with PPXY motifs on proteins (40–42). Because Spry2 does not contain a PPXY motif, we screened a number of mutant forms of Spry2 in which the proline-rich regions or the C-terminal, SH3 domain binding region on Spry2 were substituted. None of these mutations on Spry2 altered the association with Nedd4 (supplemental Fig. S2). Besides Tyr<sup>35</sup>, Spry2 is phosphorylated on several Ser/Thr residues (15, 37, 38, 43, 44). Among these, phosphorylation of Ser<sup>112</sup> and Ser<sup>121</sup> have been implicated in regulating the stability of Spry2 (38). Substitution of these Ser residues with Ala on Spry2 abrogated its association with Nedd4 (Fig. 2B) and, consistent with the role of these residues as phosphorylation sites, the S112A/S121A mutant of Spry2 had increased mobility on SDS-PAGE (Fig. 2B). These data show that the phosphorylation of Ser<sup>112</sup>/Ser<sup>121</sup> on Spry2 is likely important for interaction between Spry2 and Nedd4 and explains the interactions of Nedd4 with the slower migrating band of Spry2. Although enhanced Ser/Thr phosphorylation of Spry2 has been shown after overexpression of epidermal growth factor receptor (38), we did not observe any significant changes in mobility of the Spry2 bands with and without EGF or serum. This suggests that Ser<sup>112</sup> and Ser<sup>121</sup> sites are phosphorylated in our experiments under all conditions, thus, explaining the constitutive interactions between Nedd4 and Spry2 in the absence of agonists (Figs. 1A and supplemental S2).

A majority of interactions between HECT family ubiquitin ligases and their associated proteins involve WW domains. To determine whether the WW domains on Nedd4 are involved in the interaction with Spry2, pulldown experiments with GST fusion proteins of the WW domains were performed. GST fusion proteins comprising WW domains 1–3, as well as WW domains 1 and 2, interacted with Spry2 (Fig. 2C). However, the GST-WW domain 3 did not pull down Spry2, and the amount of Spry2 pulled down by GST-WW domains 1–2 were lower than that observed with GST-WW domains 1–3 (Fig. 2C). Additionally, GST-WW domains 1–3 of Nedd4 pulled down significantly more wild-type Spry2 as compared with its S112A/S121A mutant (Fig. 2D and E). In similar experiments, GST-WW domains 1–4 of human Nedd4-1 also interacted with wild-type Spry2 or its Y55F mutant, but not with its S112A/S121A mutant (supplemental Fig. S3A). To determine whether Spry2 interacted with WW domains of other HECT domain family E3 ubiquitin ligases, we investigated the ability of GST-WW domains 1–4 of AIP4 to pull down Spry2. As
reported previously (45), GST-AIP4 WW domains 1–4 pulled down H9252-arrestin 2/3 (data not shown). However, Spry2 did not interact with AIP4 WW domains (data not shown). Moreover, in experiments where we overexpressed full-length AIP4 with Spry2 in HEK293T cells and immunoprecipitated AIP4, we did not detect any Spry2 in the immunocomplex (data not shown). These findings demonstrate Spry2 does not interact with all HECT domain ubiquitin ligases that contain WW domains.

To determine whether Nedd4 interacted with the other isoforms of Spry, Nedd4 was coexpressed with FLAG-tagged Spry isoforms and in IPs of Nedd4, the co-IP of Spry isoforms was monitored. Although the Ser residue equivalent to Ser^{112} on Spry2 is conserved in all isoforms of Spry, Ser^{121} on Spry2 is conserved only on Spry1; Spry3 and Spry4 have Met and Val residues, respectively, in this position. Given this difference and consistent with our findings that Ser^{112} and Ser^{121} on Spry2 are necessary for interaction with Nedd4 (Fig. 2, B–E), only Spry1 and Spry2 interacted with Nedd4 (Fig. 2F). Thus, for interactions between Spry2 and Nedd4, Ser^{112} and Ser^{121} on Spry2 as well as the WW domains on Nedd4 are critical.

**Phosphorylation of Ser^{112}/Ser^{121} in Spry2 Is Necessary for Its Interactions with Nedd4**—Because Ser^{112} and Ser^{121} on Spry2 have been reported to be phosphorylated by Mnk1 (38), we used three approaches to investigate whether the interactions between Spry2 and Nedd4 were dependent upon the phosphorylation status of these two Ser residues. First, we treated HEK293T cells expressing HA-tagged Spry2 with a concentration (20 μM) of the Mnk1/Mnk2 inhibitor CGP57380 (46), which was empirically determined to show a significant decrease in the upper band and an increase in the lower band without altering total Spry2 content (data not shown). Lysates from these cells were subjected to pulldown assays using GST-WW1–3 Nedd4. As shown in Fig. 3A, GST-WW1–3 Nedd4 interaction with Spry2 was markedly diminished when cells were treated with the Mnk1 inhibitor. As a second approach, we silenced Mnk1 or Mnk2 using specific siRNAs. As shown in Fig. 3B, although Mnk1 was efficiently silenced, neither the migration of Spry2 nor the association of Spry2 with Nedd4 were altered. In contrast, silencing of Mnk2 decreased the intensity of the upper band and increased the lower Spry2 band (Fig. 3B). Additionally, silencing of Mnk2 decreased the interactions between Spry2 and Nedd4. As a third approach, we substituted Ser^{112} and Ser^{121} on Spry2 with either Asp or Glu residues to mimic phosphorylations at these sites. Lysates of cells transfected with wild-type and mutant forms of Spry2 harboring substitutions at Ser^{112} and Ser^{121} were subjected to pulldown assays with GST-WW1–3 Nedd4. Although the wild-type, S112E/S121E, and S112E/S121D forms of Spry2 were pulled down by the GST-WW1–3 Nedd4, interaction of the S112E/S121D form of Spry2 with GST-WW1–3 Nedd4 was weaker than that of the wild-type Spry2 or its S112E/S121E form (Fig. 3, C and D).
Regulation of Sprouty2 by Nedd4

S112A/S121A mutant of Spry2 was not pulled down to any significant extent; the same was the case with the S112D/S121D and S112D/S121E mutant forms of Spry2 (Fig. 3, C and D). These data demonstrate that interactions between Nedd4 and Spry2 are dependent on the phosphorylation status of Ser112 and Ser121, and that substitution of these residues by Glu may more closely mimic phosphorylations at these positions. Notably, the S112E/S121E phosphomimetic form of Spry2 did not interact with GST-AIP4 WW domains 1–4 (data not shown), further demonstrating that not all WW domains of the HECT domain family ubiquitin ligases interact with Spry2. Importantly, the data in Fig. 3B suggest that Mnk2 and not Mnk1 is the kinase responsible for phosphorylation of Ser112/Ser121.

Nedd4 Ubiquitinitates Spry2—To determine whether Spry2 is a substrate for Nedd4, we first investigated the role of endogenous Nedd4 in the ubiquitination of Spry2. Silencing of endogenous Nedd4 in HEK293T cells transfected with FLAG-tagged ubiquitin significantly decreased the ubiquitination of Spry2 (Fig. 4A). As expected, overexpression of Nedd4 markedly increased the ubiquitination of Spry2 (Fig. 4B). The ubiquinated protein bands in the IPs of Spry2 in the presence and absence of Nedd4 could be due to ubiquitinated protein(s) that associate with Spry2 rather than Spry2 itself. To address this possibility, prior to IP of Spry2, cell lysates were treated with 2% SDS and boiled for 10 min to dissociate any proteins that may be bound to Spry2. The SDS-containing samples were then diluted 1:15 in the IP buffer and Spry2 was immunoprecipitated. This method has previously been documented to remove associated proteins from the immunoprecipitated protein of interest (47). As shown in Fig. 4C, despite SDS treatment and boiling, Nedd4 expression increased the ubiquitination of Spry2, indicating that Spry2, and not any associated protein(s), is ubiquitinated. Moreover, treatment of cells with MG132 to inhibit proteosomal degradation of ubiquitinated proteins and increase their accumulation also elevated the amount of ubiquitinated Spry2, but blunted the difference in the presence and absence of Nedd4 (supplemental Fig. S4). Importantly, it should be noted that Spry2 appears to be polyubiquitinated by Nedd4, explaining the multiple ubiquitinated bands (Fig. 4A–C). The Nedd4-mediated polyubiquitination of Spry2 involves Lys48 and not Lys63 on ubiquitin (supplemental Fig. S5). Polyubiquitination of proteins involving Lys48 on ubiquitin targets them for proteosomal degradation (48). Hence, as suggested by our initial data with wild-type and catalytically inactive Nedd4 (see supplementary Fig. S1), Spry2 is not only a substrate for Nedd4 but, consistent with its increase with MG132, is polyubiquitinated via Lys48 on ubiquitin and targeted for proteosomal degradation.

Nedd4 Regulates the Stability of Spry2—Nedd4 polyubiquitinitates Spry2 via Lys48 on ubiquitin (supplemental Fig. S5). This type of polyubiquitination targets proteins for proteosomal degradation (49). Therefore, we next investigated whether or not silencing of endogenous Nedd4 or overexpression of Nedd4 altered the stability of endogenous and overexpressed Spry2 and its S112A/S121A mutant that does not associate with Nedd4. Silencing of endogenous Nedd4 increased the stability of endogenous Spry2 in HEK293T cells (Fig. 5, A and D). This is consistent with the decrease in Spry2 ubiquitination when endogenous Nedd4 is silenced (Fig. 4A). Expression of the wild-type Nedd4 decreased the cellular content of endogenous Spry2 that did not change significantly after CHX treatment over the time course studied (Fig. 5B). The possible reasons for this observation are discussed later. On the other hand, consistent with the role of catalytically inactive Nedd4 as a dominant-negative (39), expression of the C867S mutant form of Nedd4 stabilized endogenous Spry2 (cf. Fig. 5, B and D). Furthermore, consistent with the lack of an interaction between the S112A/S121A mutant of Spry2 and Nedd4, the stability of S112A/S121A Spry2 was not markedly altered by overexpression of wild-type Nedd4 (Fig. 5, C and E). These data show that Nedd4 regulates the stability of endogenous and overexpressed wild-type Spry2, but not its S112A/S121A mutant that does not interact with Nedd4.
Regulation of Sprouty2 by Nedd4

Nedd4 by Regulating Cellular Spry2 Levels Modulates FGF Signaling—It is now well established that Spry2 antagonizes the biological actions of FGF, in part by inhibiting FGF-mediated activation of ERK1/2 (43, 50–53). This inhibition of ERK1/2 signaling via the FGF receptor involves binding of Grb2 to the C-terminal Pro-rich region (Pro304–Arg309) of Spry2 (52). To determine the functional role of Nedd4-mediated regulation of cellular Spry2 content, we investigated whether or not manipulations of Nedd4 content and, therefore, cellular Spry2 levels, regulated the ability of FGF to activate ERK1/2. For this purpose, HeLa cells expressing modest amounts of Spry2 were utilized. As shown in Fig. 6, A and B, silencing of Nedd4 in these cells increased the cellular content of Spry2 and decreased the ability of FGF (100 ng/ml) to activate ERK1/2. Maximal activation of the FGF-elicited ERK1/2 activation was observed 10 min after addition of FGF (Fig. 6, A and B). Therefore, in subsequent experiments, ERK1/2 activation was monitored 10 min after the addition of different concentrations of FGF. As observed previously, shRNA-mediated silencing of Nedd4 increased the amount of Spry2 and decreased the ability of different concentrations of FGF to activate ERK1/2 (Fig. 6, A–C). That the decrease in FGF-mediated ERK1/2 activation upon Nedd4 silencing was the result of an increase in cellular Spry2 content is shown by the data in Fig. 6, D and E. Hence, silencing of Spry2 in cells that had been treated with shRNA against Nedd4 resulted in an increase in FGF-mediated activation of ERK1/2. These latter data confirm that the alterations in FGF signaling when Nedd4 is silenced are due to changes in Spry2 content.

Silencing of Mnk2, but Not Mnk1, Increases Spry2 Content and Regulates FGF Signaling—Mnk2 silencing decreases the amount of phospho-Spry2 and its interactions with Nedd4 (Fig. 3B). The lack of this interaction should be akin to Nedd4 silencing and augment the ability of Spry2 to inhibit FGF signaling. Indeed, when Mnk2 was silenced, the ability of Spry2 to inhibit FGF-elicited ERK1/2 activation was markedly augmented (Fig. 7). Mnk1 silencing did not affect the inhibition of ERK1/2 activation by Spry2 (Fig. 7).

DISCUSSION

In this report, we describe a novel interaction between Nedd4 and Spry2 and provide evidence showing that endogenous Nedd4 can ubiquitinate Spry2 and regulate its cellular content that then modulates the ability of the FGF receptor to activate downstream signaling. This is the first demonstration of the regulation of a Spry family member by a HECT domain E3 ubiquitin ligase. The interaction between Spry2 and Nedd4 also requires Ser112 and Ser121 on Spry2 (52). To determine the functional role of Nedd4-mediated regulation of cellular Spry2 content, we investigated whether or not manipulations of Nedd4 content and, therefore, cellular Spry2 levels, regulated the ability of FGF to activate ERK1/2. For this purpose, HeLa cells expressing modest amounts of Spry2 were utilized. As shown in Fig. 6, A and B, silencing of Nedd4 in these cells increased the cellular content of Spry2 and decreased the ability of FGF (100 ng/ml) to activate ERK1/2. Maximal activation of the FGF-elicited ERK1/2 activation was observed 10 min after addition of FGF (Fig. 6, A and B). Therefore, in subsequent experiments, ERK1/2 activation was monitored 10 min after the addition of different concentrations of FGF. As observed previously, shRNA-mediated silencing of Nedd4 increased the amount of Spry2 and decreased the ability of different concentrations of FGF to activate ERK1/2 (Fig. 6, A–C). That the decrease in FGF-mediated ERK1/2 activation upon Nedd4 silencing was the result of an increase in cellular Spry2 content is shown by the data in Fig. 6, D and E. Hence, silencing of Spry2 in cells that had been treated with shRNA against Nedd4 resulted in an increase in FGF-mediated activation of ERK1/2. These latter data confirm that the alterations in FGF signaling when Nedd4 is silenced are due to changes in Spry2 content.

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Regulation of Sprouty2 by Nedd4

![Regulation of Sprouty2 by Nedd4](image)

of Ser residues on Spry2 increases its interactions with B-Raf, with Ser112/Ser121 being the main determinants of this interaction (54). Thus, upon phosphorylation of Ser112/Ser121, Spry2 would switch its interactions from B-Raf to Nedd4.

Although the interaction between Spry2 and Nedd4 requires phosphorylation of Ser112/Ser121, we did not observe any significant changes in the co-immunoprecipitation of these proteins before or after treating cells with EGF or serum. Apparently, therefore, even when cells are grown under serum-free condi-

tions, there is a sufficient amount of Spry2 that is phosphorylated on Ser112/Ser121. This is supported by our observations that unlike studies conducted with overexpressed EGF receptors (38) and FGF receptors (37), we did not observe any significant change in mobility of the Spry2 protein on polyacrylamide gels either with or without growth factor or serum (see e.g. supplemental Fig. S2). However, the Mnk1/2 inhibitor and silencing of Mnk2 significantly decreased the upper Spry2 band and also diminished the ability of Spry2 to interact with Nedd4 (Fig. 3A).

The WW domains of HECT family ubiquitin ligases bind Pro-rich regions and phospho-Thr/phospho-Ser sites followed by a Pro residue (reviewed in Ref. 55). Nedd4 WW domains bind PPXY motifs with high affinity and with somewhat lower affinity to phospho-Thr/ phospho-Ser preceding a Pro motif (56). Spry2 does not contain a PPXY motif and substitutions of different Pro-rich regions did not alter the association with Nedd4 (supplemental Fig. S2). Moreover, there are no Pro residues in the region encompassed by Ser112/Ser121 on Spry2. Thus, binding of the Nedd4 WW domain to Spry2 appears to involve a non-canonical, phospho-Ser-containing, WW domain binding site. A recent report showed that the WW domains of AIP4 can interact with a phosphoserine-containing region in the C terminus of the CXCR4 chemokine receptor, which also does not contain any Pro residues (57). As with Spry2/Nedd4, the interaction between the CXCR4 receptor and AIP4 was dependent on the phosphorylation of two Ser residues in this region (57). Comparison of the regions surrounding phospho-Ser residues on CXCR4 and Spry2 that bind the WW domains of AIP4 and Nedd4, respectively, suggest that the consensus sequence of this motif would be SXXSSXXXXXS. Although the first and last Ser residues (Ser112/Ser121) in this motif are phosphorylated on Spry2, the two Ser residues in the middle of this motif are phosphorylated on CXCR4 (57). This subtle difference in phosphorylation of Ser residues within this motif could be the basis of the specificity of the interactions of Nedd4 and AIP4 WW domains with Spry2 and CXCR4, respectively.
This difference may also explain why the AIP4 WW domains do not interact with Spry2.

To date, c-Cbl and Siah2 have been demonstrated to regulate Spry2 content (7, 8, 22, 33), although the role of endogenous Siah2 in regulating Spry levels remains to be determined. Herein, we have shown that HECT domain family member Nedd4 can also polyubiquitinate Spry2 and decrease its cellular content. By silencing endogenous Nedd4 or opposing its actions by expression of the catalytically inactive (C867S), dominant-negative, form of Nedd4, we show that the rate of endogenous or overexpressed Spry2 degradation is regulated by Nedd4 (Fig. 5). Interestingly, when wild-type Nedd4 was overexpressed, as expected, the amount of endogenous Spry2 was decreased (Fig. 5B), but surprisingly, in the presence of cycloheximide, the degradation of the remaining Spry2 in the cells was fairly constant (Fig. 5B). These findings suggest that when wild-type Nedd4 is overexpressed, it diminishes the Nedd4-sensitive Spry2 content, leaving behind a cellular pool of Spry2 that is not accessible to Nedd4 that then turns over rather slowly. This other pool of Spry2 could be inaccessible to Nedd4 and regulated by other ubiquitin ligases such as c-Cbl. We also observed that in HEK293T cells, the Spry2 S112A/S121A mutant is more stable than its wild-type counterpart (Fig. 5, A and B). This is in stark contrast to the observations that in CHO-K1 cells the S112A/S121A mutant is less stable than the wild-type protein (38). This difference may be related to differences in cell types and the relative abundance of Nedd4 in the two cell types. Thus, depending upon the cell type, the abundance of E3 ligases (c-Cbl and Nedd4) and, perhaps, the Src family of tyrosine kinases that phosphorylates Tyr145, a prerequisite for c-Cbl interactions, the stability of Spry2 may be differentially regulated by phosphorylation of Ser112 and Ser121.

The functional significance of the Nedd4–mediated ubiquitination of Spry2 and, therefore, modulation of cellular Spry2 content, is underscored by our findings that silencing of Nedd4 that resulted in an increase in cellular Spry2 content was accompanied by attenuation of FGF-elicited activation of ERK1/2 (see Fig. 6). That this attenuation of ERK1/2 signaling was due to elevations in Spry2 content is shown by the findings that when Nedd4 shRNA-mediated elevation in Spry2 was attenuated by siRNA against Spry2, the FGF-mediated activation of ERK1/2 was restored (Fig. 6, D and E). Moreover, the role of Mnk2 phosphorylation of Spry2 is also demonstrated by the finding that silencing of Mnk2, which decreases Spry2/Nedd4 interactions, also augmented the ability of Spry2 to inhibit FGF signaling (Fig. 7).

As a negative regulator of FGF signaling, during embryonic development, Spry2 has been shown to be overexpressed at the sites of FGF action (58–60). In this context, Spry2 plays an important role in limb bud development (58, 61, 62). Nedd4 expression is decreased during limb bud development in the same areas as Spry2 expression is increased (63, 64). Thus, based on our findings, it is tempting to speculate the decrease in Nedd4 content during limb bud development increases Spry2 content to regulate the actions of FGF.

In conclusion, we present novel findings showing that Nedd4 WW domains interact with a non-canonical, phospho-Ser-containing motif on Spry2. The interactions between Spry2 and Nedd4 are Mnk2-dependent and involve phosphorylation of Ser112/Ser121 on Spry2. Nedd4 polyubiquitinates and regulates the cellular content of Spry2. This Nedd4-mediated regulation of Spry2 content modulates the signaling via the FGF receptor.

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