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hSpry2 Is Targeted to the Ubiquitin-Dependent Proteasome Pathway by c-Cbl

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Summary

Sprouty was originally identified in a genetic screen in *Drosophila* as an antagonist of fibroblast (FGF) and epidermal growth factor (EGF) signaling [1, 2]. Subsequently, four vertebrate homologs were discovered; among these, the human homolog Sprouty 2 (hSpry2) contains the highest degree of sequence homology to the *Drosophila* protein [3, 4]. It has been shown that hSpry2 interacts directly with c-Cbl, an E3-ubiquitin ligase, which promotes the downregulation of receptor tyrosine kinases (RTKs) [5]. In this study, we have investigated the functional consequences of the association between hSpry2 and c-Cbl. We have found that hSpry2 is ubiquitinated by c-Cbl in an EGF-dependent manner. EGF stimulation induces the tyrosine phosphorylation of hSpry2, which in turn enhances the interaction of hSpry2 with c-Cbl. The c-Cbl-mediated ubiquitination of hSpry2 targets the protein for degradation by the 26S proteasome. An enhanced proteolytic degradation of hSpry2 is also observed in response to FGF stimulation. The FGF-induced degradation of hSpry2 limits the duration of the inhibitory effect of hSpry2 on extracellular signal-regulated kinase (ERK) activation and enables the cells to recover their sensitivity to FGF stimulation. Our results indicate that the interaction of hSpry2 with c-Cbl might serve as a mechanism for the downregulation of hSpry2 during receptor tyrosine kinase signaling.

Results and Discussion

EGF Stimulates the c-Cbl-Mediated Ubiquitination of hSpry2

Signal transduction pathways that emanate from receptor tyrosine kinases (RTK) control various aspects of morphogenesis, patterning, cellular proliferation, and differentiation in both vertebrates and invertebrates [6]. It is now widely recognized that the spatial and temporal regulation of RTK signaling is accomplished by the interplay between negative and positive regulators [7]. Sprouty proteins comprise a conserved family of inducible antagonists of RTK signaling [1, 2]. *Drosophila* Sprouty is a 63 kDa protein containing a cysteine-rich domain within its C terminus that is highly conserved in the four mammalian Sprouty homologs (Spry 1–4). The N-terminal region of Sprouty proteins is highly divergent, with the exception of two small regions of similarity between the *Drosophila* and mammalian proteins [1]. One of these regions has been shown to bind to the ubiquitin ligase c-Cbl, a well-known negative regulator of RTK signaling [5]. c-Cbl mediates the multiubiquitination of receptors such as the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and colony-stimulating factor 1 receptor (CSF-1R) and thereby targets them for degradation by the proteasomal/lysosomal pathway [8–10]. It has been shown that the interaction of Spry2 with c-Cbl interferes with the ability of c-Cbl to promote the ubiquitination and degradation of the EGF receptor [5, 11, 12].

Proteins that interact with c-Cbl are often found to be ubiquitinated as a consequence of the ubiquitin ligase activity of c-Cbl. To determine whether the association between c-Cbl and hSpry2 leads to the ubiquitination of hSpry2, we compared the abilities of ectopically expressed hSpry2 and hSpry2-ΔCbl, a deletion mutant lacking the c-Cbl binding region (residues 36–65), to become ubiquitinated upon EGF stimulation. CHOK1 cells, which lack endogenous EGF receptor, were transiently transfected with expression vectors encoding EGFR, hemagglutinin (HA)-tagged ubiquitin, and HA-tagged hSpry2 constructs. As shown in Figure 1A, low levels of hSpry2 ubiquitination were detected in growth factor-deprived cells. However, the addition of EGF induced a significant increase in the ubiquitination of hSpry2, as indicated by the abundant presence of high molecular weight HA-immunoreactive bands in the anti-hSpry2 immunoprecipitates. In contrast, the hSpry2-ΔCbl mutant, which does not associate with c-Cbl, was not ubiquitinated either in the presence or in the absence of EGF (Figure 1B). These observations indicate that c-Cbl mediates the ubiquitination of hSpry2 in an EGF-dependent manner through its association with the amino-terminal region of hSpry2. The ability of c-Cbl to ubiquitinate hSpry2 does not reflect a unique aspect of our experimental system, as similar results were obtained by using COS-1 cells (data not shown). It is noteworthy that the enhanced ubiquitination of hSpry2 in response to EGF stimulation is accompanied by a decrease in the levels of hSpry2 (Figure 1A). As discussed below, this decrease reflects the enhanced degradation of hSpry2. We have observed that the immunoprecipitates of hSpry2 and hSpry2-ΔCbl both contain an HA-immunoreactive band of an apparent molecular weight of 45 kDa that is present under both serum deprivation and EGF stimulation conditions (Figures 1A and 1B). The identity of this band is presently unknown.

EGF-Induced Tyrosine Phosphorylation of hSpry2 Increases Its Association with c-Cbl

Since the ubiquitination of hSpry2 requires the interaction between hSpry2 and c-Cbl, we hypothesized that

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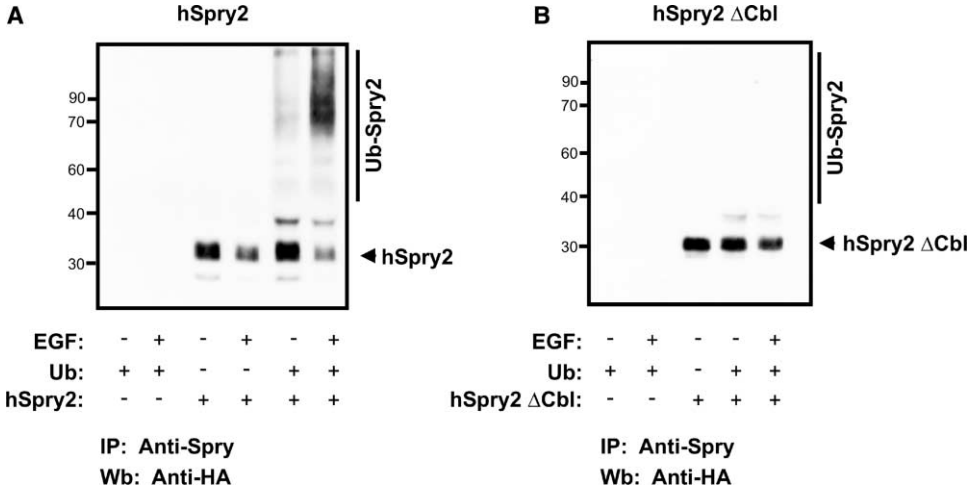


Figure 1. c-Cbl Mediates the Ubiquitination of hSpry2 in an EGF-Dependent Manner

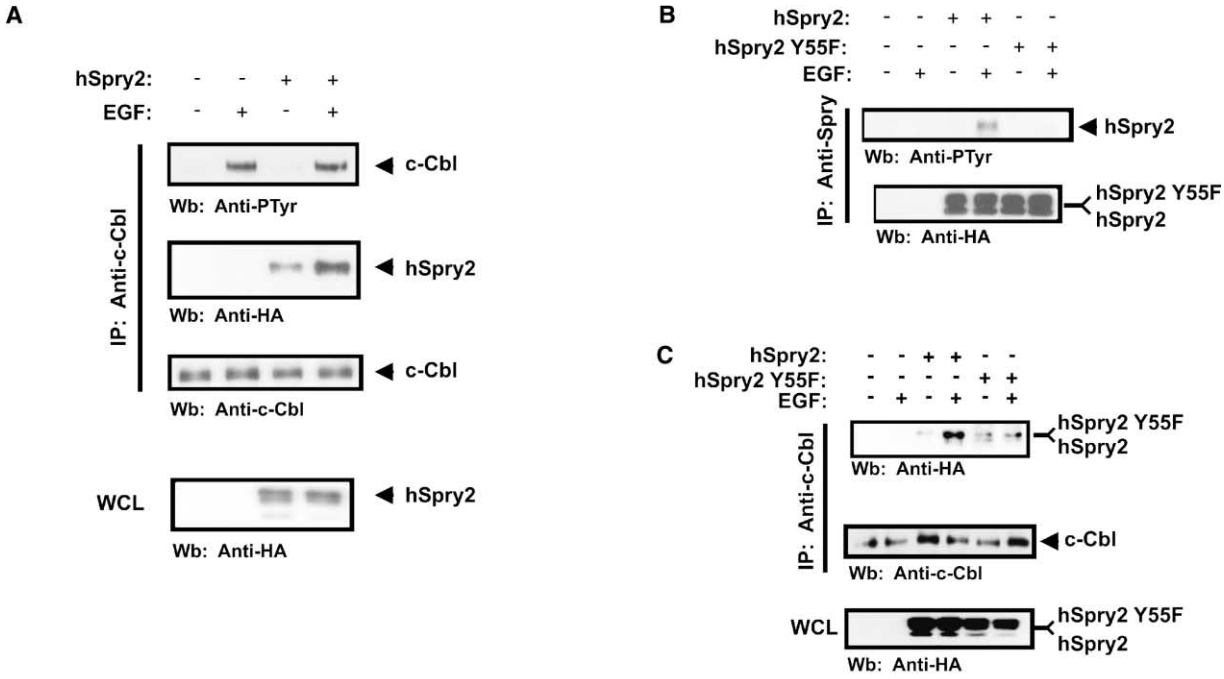
(A and B) CHOK1 cells were transiently transfected with expression vectors encoding the EGFR, HA-ubiquitin (Ub), and either (A) HA-hSpry2 or (B) HA-hSpry2-ΔCbl. A total of 24 hr posttransfection, the cells were serum starved for 16 hr and then incubated with (+) or without (−) EGF (100 ng/ml) for 10 min. Subsequently, the cells were lysed and analyzed by immunoprecipitation (IP) and Western blotting (Wb) with the indicated antibodies. Each experiment shown is representative of at least three independent experiments.

the stimulation of hSpry2 ubiquitination by EGF might result from a growth factor-dependent increase in the binding affinity between c-Cbl and hSpry2. To test this prediction, we compared the interaction between hSpry2 and c-Cbl in the presence and absence of EGF in CHOK1 cells that were transiently transfected with EGFR and HA-tagged hSpry2. Consistent with earlier findings [5], hSpry2 was able to constitutively interact with c-Cbl (Figure 2A). Upon EGF addition, the interaction between hSpry2 and c-Cbl was significantly enhanced (Figure 2A). Thus, the increase in the ubiquitination of hSpry2 seen after EGF stimulation may in part be mediated by the EGF-induced increase in hSpry2-c-Cbl interaction. It should be noted that the increase in the association of hSpry2 with c-Cbl following EGF stimulation could not be detected when the levels of ectopically expressed hSpry2 were high (data not shown). This may explain why a modulation of association between hSpry2 and c-Cbl by growth factors was not detected in an earlier study [5].

To identify the determinants within hSpry2 that are responsible for the EGF-dependent increase in its binding affinity toward c-Cbl, we focused our attention on a conserved tyrosine residue of hSpry2, tyrosine 55, that is located within the c-Cbl binding region of hSpry2 [13]. To establish whether tyrosine 55 is phosphorylated upon EGF stimulation, CHOK1 cells were transiently transfected with the EGF receptor and HA-tagged hSpry2 or an hSpry2 mutant in which tyrosine 55 was mutated to phenylalanine (hSpry2Y55F). As shown in Figure 2B, hSpry2 became tyrosine phosphorylated in response to EGF stimulation. In contrast, the hSpry2Y55F mutant failed to undergo tyrosine phosphorylation upon EGF stimulation (Figure 2B), suggesting that this residue is critical for the EGF-induced tyrosine phosphorylation of hSpry2.

Next, we utilized the hSpry2Y55F mutant to investigate the role of hSpry2 tyrosine phosphorylation in c-Cbl binding. CHOK1 cells were transfected with expression vectors encoding EGFR and HA-hSpry2 or hSpry2Y55F, and endogenous c-Cbl was immunoprecipitated. As illustrated in Figure 2C, hSpry2Y55F retained its ability to interact with c-Cbl, but, in contrast to the wild-type hSpry2, this interaction was only slightly enhanced by EGF treatment. These results indicate that the phosphorylation of tyrosine 55 of hSpry2 contributes to the regulation of the interaction between hSpry2 and c-Cbl. Consistent with this interpretation, we have found that c-Cbl-mediated ubiquitination of hSpry2Y55F after EGF stimulation is attenuated (data not shown). In a recent study, it has been shown that substitution of tyrosine 55 with alanine yields a dominant-negative mutant of hSpry2, which selectively enhances FGF-induced, but not EGF-induced, MAP kinase activation [13]. The extent to which these effects can be attributed to changes in c-Cbl binding remains to be determined.

To examine the spatial relationship between hSpry2 and c-Cbl, we have analyzed the subcellular localization of ectopically expressed HA-hSpry2 and endogenous c-Cbl in serum-starved and EGF-stimulated cells. For this analysis, we have used COS-1 cells because endogenous c-Cbl is expressed in these cells at levels that are high enough to be detected by indirect immunofluorescent staining. We have confirmed that the biochemical features of the interaction between c-Cbl and hSpry2 in COS-1 cells are identical to those observed in CHOK1 cells (data not shown). As illustrated in Figure 2D, hSpry2 is associated predominantly with vesicular structures distributed throughout the cytoplasm both in EGF-treated and untreated cells. A subpopulation of the hSpry2-containing structures was found to colocalize with the early endosome marker Rab5 [14], and this finding suggests some degree of functional overlap between hSpry2 and the endocytic pathway (data not shown). The vesicular pattern of hSpry2 subcellular distribution was observed in a number of different cells



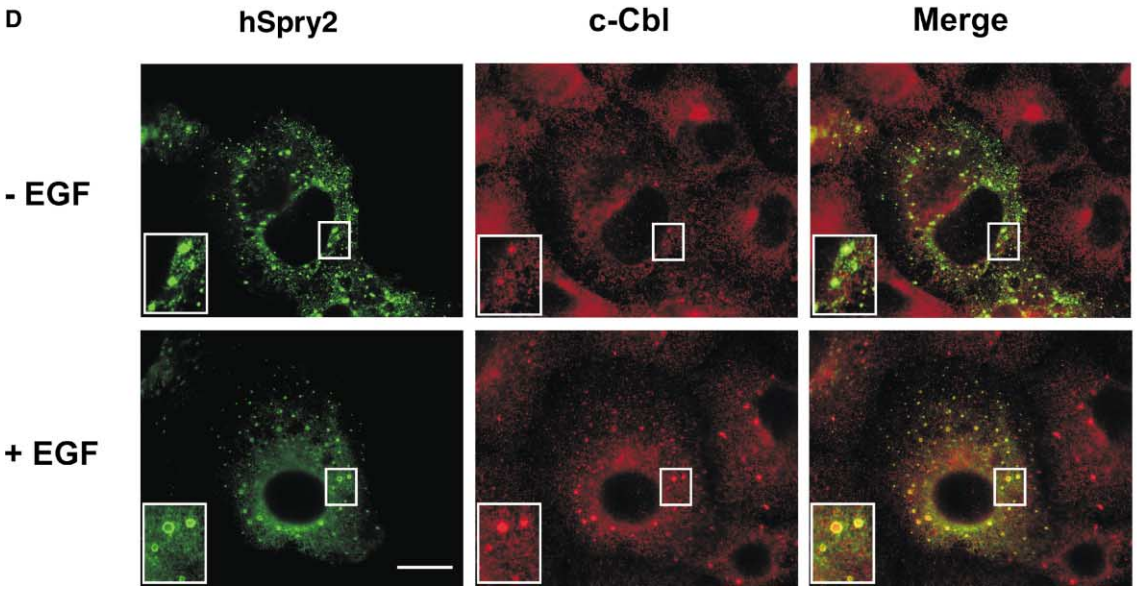


Figure 2. EGF Stimulation Enhances the Association of hSpry2 with c-Cbl

(A–C) Tyrosine phosphorylation of hSpry2 modulates the interaction with c-Cbl. CHOK1 cells were transiently transfected with expression plasmids encoding the EGFR and either (A–C) HA-hSpry2 or (B and C) HA-hSpry2Y55F. Cells were starved and stimulated with EGF as described in the legend of Figure 1. Subsequently, the cells were lysed and analyzed by immunoprecipitation (IP) and Western blotting (Wb) with the indicated antibodies. Protein expression levels in whole-cell lysates (WCL) were determined by immunoblotting. The experiments shown are representative of three independent experiments.

(D) Subcellular distribution of c-Cbl and HA-hSpry2. COS-1 cells transfected with hSpry2 were stimulated with EGF (100 ng/ml) for 15 min and were processed for immunofluorescenet staining as described in the Supplementary Experimental Procedures (see the Supplementary Material available with this article online). The cells were analyzed by laser scanning confocal microscopy to detect colocalization (yellow) of HA-hSpry2 (green) and c-Cbl (red). Insert boxes show enlarged areas corresponding to specific examples of the extent of hSpry2/c-Cbl colocalization. The scale bar represents 10 μM.

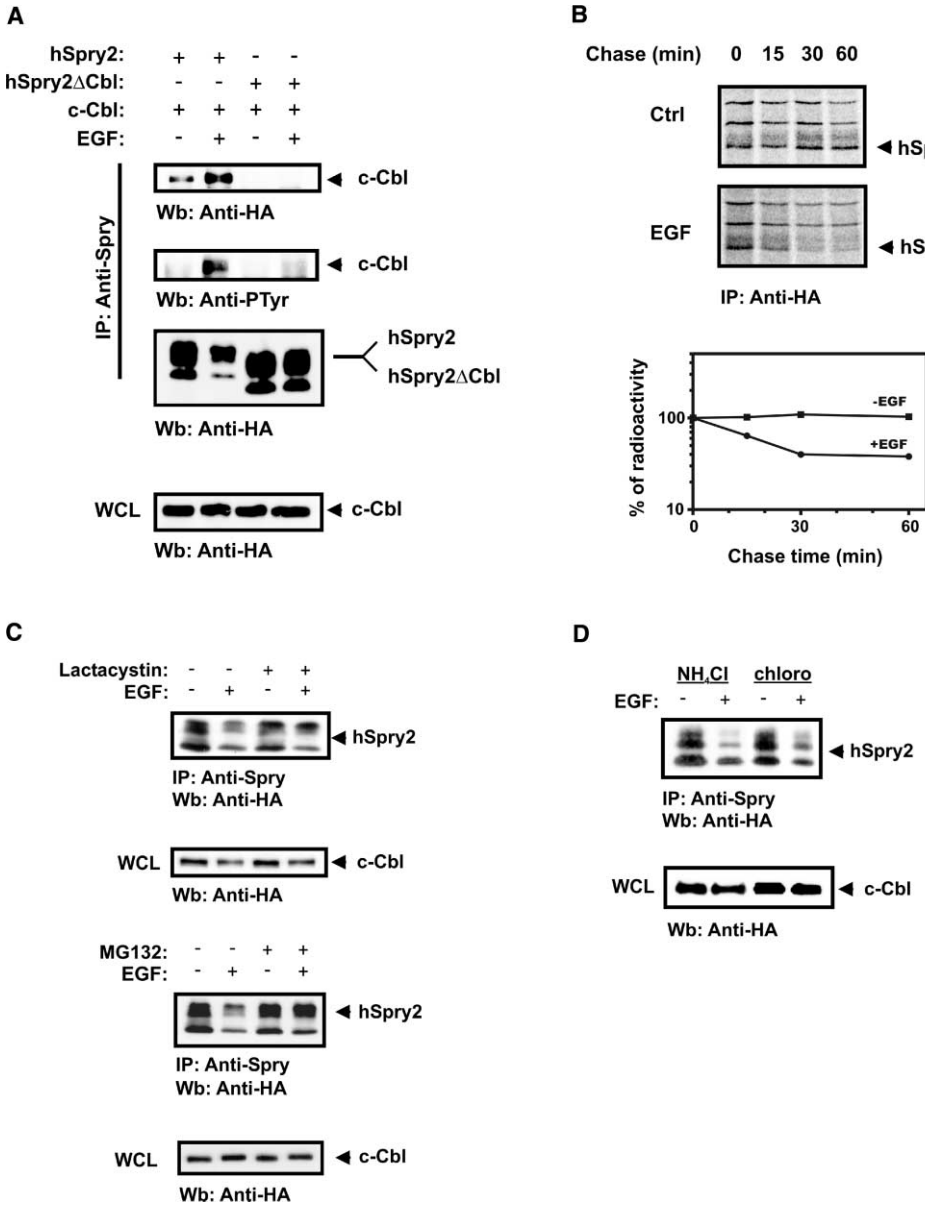


Figure 3. c-Cbl-Dependent Ubiquitination Stimulates the Degradation of hSpry2 by the 26S Proteasome Pathway

(A) CHOK1 cells were transiently transfected with expression plasmids encoding EGFR, HA-c-Cbl, and either HA-hSpry2 or HA-hSpry2-ΔCbl. Serum starved cells were incubated with (+) or without (−) EGF as described in Figure 1.

(B) CHOK1 cells were transiently transfected with expression plasmids encoding EGFR, HA-c-Cbl, and HAM-hSpry2. Pulse-chase analysis of hSpry2 stability was performed as described in the Supplementary Experimental Procedures. Where indicated, EGF (100 ng/ml) was added to the chasing medium. Autoradiograms were quantified with a Storm Phosphor Imager (Molecular Dynamics).

(C) CHOK1 cells were transiently transfected with expression plasmids encoding the EGFR, HA-c-Cbl, and HA-hSpry2. A total of 24 hr posttransfection, the cells were serum starved for 16 hr and were then treated with lactacystin (20 μM), MG-132 (42 μM), or DMSO as a control for 2 hr. The cells were then stimulated with EGF (100 ng/ml) for 10 min in the presence or absence of lactacystin or MG-132.

(D) CHOK1 cells were transiently transfected with expression plasmids encoding the EGFR, HA-c-Cbl, and HA-hSpry2. A total of 24 hr posttransfection, the cells were serum starved for 16 hr in the presence of NH_4Cl (20 mM) or chloroquine (chloro) (100 μM). The cells were then stimulated with EGF (100 ng/ml) for 10 min in the presence of NH_4Cl or chloroquine. For (A), (C), and (D), following EGF stimulation, the cells were lysed and analyzed by immunoprecipitation (IP) and Western blotting (Wb) with the indicated antibodies. Protein expression levels in whole-cell lysates (WCL) were determined by immunoblotting. For (A)–(D), the experiments shown are representative of three independent experiments.

types (data not shown), and, for reasons that are presently unknown, it does not resemble the cytoskeletal/membrane ruffles localization pattern of hSpry2 reported in an earlier study [15].

In agreement with published data [8], we have observed that c-Cbl is present in small punctate cytoplasmic structures in nonstimulated cells but redistributes into a larger vesicular structures upon EGF

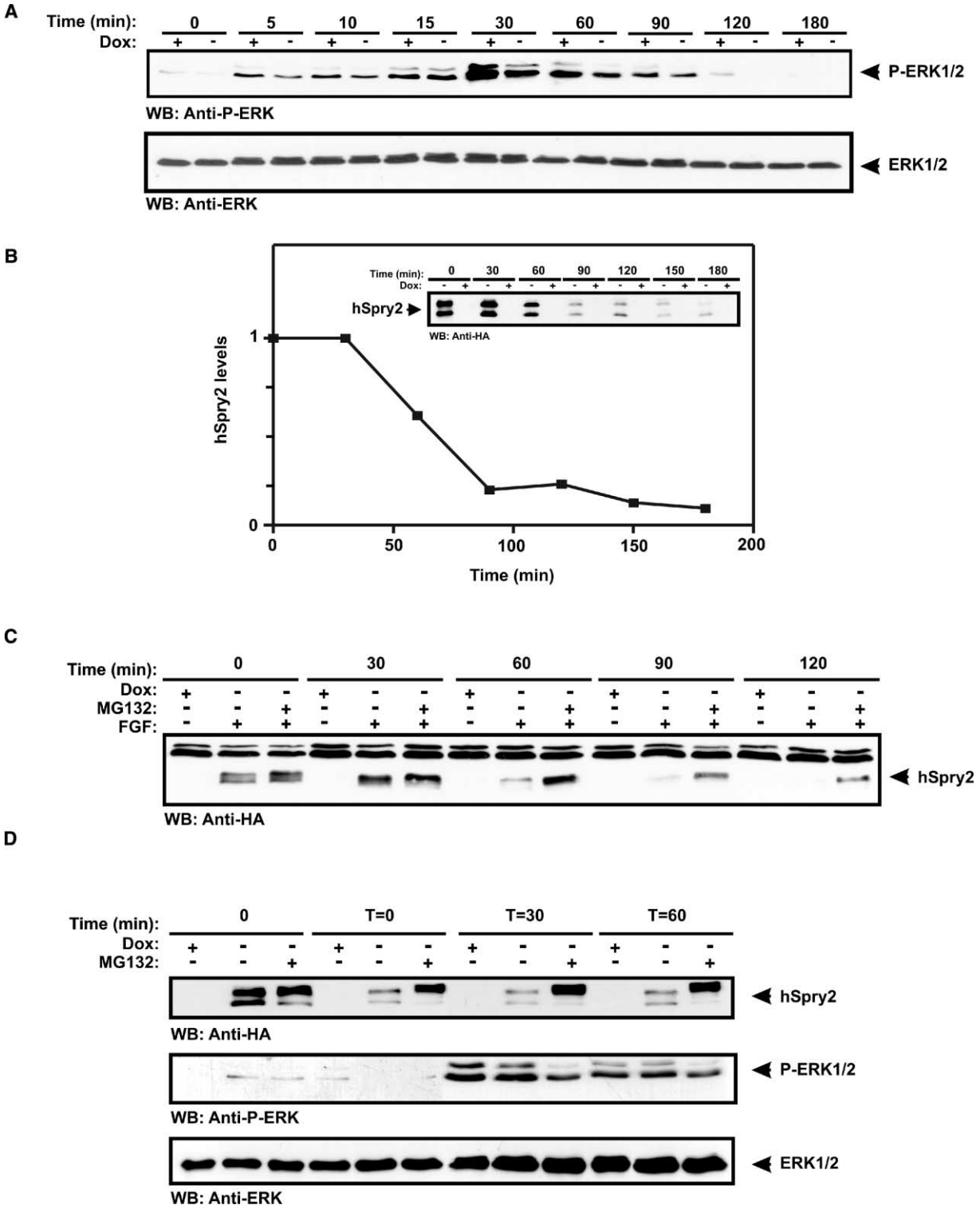


Figure 4. The Effects of hSpry2 Proteolytic Degradation on FGF-Induced ERK Activation

(A) NIH3T3 cells were maintained in the presence (+) or absence (−) of doxycylin (Dox) to repress or induce, respectively, HA-hSpry2 expression. Cells were serum starved for 12 hr and were treated with FGF-heparin (10–500 ng/ml) for the indicated intervals. Cell lysates were immunoblotted with anti-phospho-ERK1/2 antibodies and anti-ERK1/2 antibodies.

(B) NIH3T3 cells maintained in the presence (+) or absence (−) of Dox were stimulated with FGF for the indicated intervals as described in the legend of (A). Cell lysates were immunoblotted with anti-HA antibodies, and the relative intensities of the HA-hSpry2 bands were determined by densitometry scanning and are presented as arbitrary units.

(C) NIH3T3 cells maintained in the presence (+) or absence (−) of Dox were stimulated with FGF for various intervals as described in the

stimulation (Figure 2D). The presence of hSpry2 did not alter the subcellular distribution pattern of c-Cbl in non-stimulated cells, and, under these conditions, only a small fraction of c-Cbl colocalized with hSpry2. In contrast, in EGF-stimulated cells, there was a significant increase in the amount of c-Cbl associated with hSpry2-containing structures (Figure 2D). This finding is consistent with the biochemical data showing a ligand-dependent increase in binding of c-Cbl to hSpry2 (Figure 2A). The vesicular structures containing both hSpry2 and c-Cbl were devoid of early or late endosomal markers (data not shown). These observations suggest that the interaction of c-Cbl with hSpry2 leads to the sequestration of c-Cbl away from the endocytic pathway, which might contribute to the capacity of hSpry2 to inhibit c-Cbl-mediated ubiquitination and degradation of the EGF receptor described previously [11, 12].

Ubiquitinated hSpry2 Is Degraded by 26S Proteasome Pathway

The most commonly known function of ubiquitination is to target proteins to the 26S proteasome for degradation [16]. Since, the ubiquitination of hSpry2 is augmented by EGF, one would predict that EGF stimulation would result in the enhanced degradation of hSpry2. We have observed that EGF stimulation was accompanied by a reduction in the amount of hSpry2 (Figure 1A). In order to confirm that the observed reduction is dependent on the interaction of Sprouty with c-Cbl, CHOK1 cells were transiently transfected with EGFR, HA-tagged c-Cbl, and either HA-tagged hSpry2 or hSpry2-ΔCbl. As illustrated in Figure 3A, there was a significant decrease in the amount of hSpry2 present in immunoprecipitates derived from EGF-stimulated cells. In contrast, the levels of hSpry2-ΔCbl were not altered by the addition of EGF, indicating that c-Cbl interaction is required for the EGF-induced reduction in hSpry2 abundance (Figure 3A).

To determine whether observed reduction of hSpry2 levels reflects the enhanced degradation of Sprouty, we performed pulse-chase analysis. Because of the low methionine content of hSpry2, we employed an expression vector in which hSpry2 is fused N-terminally to the HAM tag, a methionine-rich variant of the HA tag [17]. CHOK1 cells were transiently transfected with EGFR, c-Cbl, and HAM-hSpry2. As shown in Figure 3B, in the absence of EGF, hSpry2 levels were stable and remained unchanged during the chasing time. In contrast, in EGF-treated cells, the half-life of hSpry2 decreased to $\sim$25 min. Taken together, these data demonstrate that EGF receptor activation accelerates the degradation of hSpry2 and that this process depends on the ability of hSpry2 to interact with c-Cbl.

Multiubiquitinated proteins are usually directed to the 26S proteasome pathway, although, in some cases, ubiquitinated proteins are degraded by the endosomal/lysosomal pathway [18]. To determine which degradation pathway is engaged in the proteolytic degradation of hSpry2 following EGF stimulation, we tested the effect of the 26S proteasome inhibitors lactacystin and MG-132 or the lysosomal inhibitors chloroquine and NH_4Cl on the levels of hSpry2. As shown in Figure 3C, the EGF-induced reduction in hSpry2 levels was abrogated in the presence of both 26S proteasome inhibitors. However, neither chloroquine nor NH_4Cl prevented the degradation of hSpry2 following EGF stimulation (Figure 3D). Together, these observations indicate that c-Cbl-mediated ubiquitination of hSpry2 targets hSpry2 to the proteasomal degradation pathway. Since *Drosophila* Sprouty has also been shown to interact with c-Cbl [5], it is likely that the c-Cbl-directed ubiquitination and degradation of Sprouty represents a conserved mechanism to modulate Sprouty's function.

Genetic and biochemical studies indicate that Sprouty is a component of a negative feedback loop that is set up to downregulate RTK signaling [19]. Indeed, it has been demonstrated that Sprouty expression induces the attenuation or inhibition of ERK activation by several RTKs [1, 2, 10, 13, 19–21]. Considering this mode of action, the ligand-dependent ubiquitination and degradation of Sprouty might serve to limit the duration of Sprouty activity, thereby allowing cells to regain responsiveness to RTK activation. To test this hypothesis, we have investigated the consequences of hSpry2 degradation on the temporal pattern of FGF-induced ERK activation. The inhibitory effect of Sprouty on this signaling pathway has been well documented both in *Drosophila* and in mammalian cells [1, 3, 13, 21]. As an experimental system, we have used NIH3T3 cells in which the expression of HA-hSpry2 is under the control of a tetracycline-repressible activator and hence can be induced by the removal of doxycyclin from the growth medium (Figure 4B). In agreement with other reports [13, 21], the expression of HA-hSpry2 resulted in the attenuation of both the intensity and duration of FGF-induced ERK activation (Figure 4A). This effect became apparent at 30 min after FGF stimulation and persisted up to 2 hr after stimulation. Concomitantly, the levels of HA-hSpry2 declined and reached approximately 10% of the initial levels of expression observed in the absence of FGF stimulation within 2 hr of FGF stimulation (Figure 4B). To determine whether the FGF-induced decrease in the amounts of HA-hSpry2 reflects enhanced proteolytic degradation, cells were stimulated with FGF in the presence or absence of MG-132. As illustrated in Figure 4C, the addition of MG-132 prevented to a large extent the FGF-induced decrease in the levels of HA-hSpry2. The similarity between the protective effects of MG-132 on FGF- and EGF-induced reduction in the amounts of HA-hSpry2 indicates that ligand-induced degradation of hSpry2 might be a common phenomenon shared by different

legend of (A). Where indicated, MG-132 (42 μM) was added to the culture medium 2 hr prior to the addition of FGF and was kept in the medium throughout the time course of FGF stimulation.

(D) NIH3T3 cells were treated with MG-132 and were stimulated with FGF as described in the legend of (C). A total of 2 hr after the initial stimulation with FGF (T = 0), cells were stimulated again with FGF for 30 (T = 30) and 60 (T = 60) min, and ERK activity was determined by immunoblotting of cell lysates with anti-phospho-ERK1/2 antibodies. The results in each panel are representative of at least two independent experiments.

RTKs. It should be noted that the addition of MG-132 caused an upshift in the electrophoretic mobility of HA-hSpry2. The molecular basis for this change is presently under investigation.

We took advantage of the ability of MG-132 to prevent the FGF-induced degradation of HA-hSpry2 to examine whether the decrease in the levels of HA-hSpry2 in response to an initial FGF stimulus is necessary in order to confer on cells the ability to respond to a subsequent FGF stimulus. To this end, cells were maintained for 120 min after the initial FGF stimulus in the presence or absence of MG-132 and were then restimulated with FGF. As shown in Figure 4D, in untreated cells, the levels of HA-hSpry2 were low, and ERK activation in response to the second FGF stimulus did not differ from that observed in cells that were not induced to express hSpry2. In contrast, in MG-132-treated cells, HA-hSpry2 expression was maintained at levels that are comparable to those found prior to FGF stimulation, and ERK activation in response to the second FGF stimulus was attenuated. Treatment with MG-132 had no apparent effect on FGF-induced ERK activation in the absence of hSpry2 expression (see Figure S1 in the Supplementary Material available with this article online). Thus, by preventing hSpry2 from persisting for prolonged periods after RTK activation, the ligand-dependent degradation of hSpry2 enables cells to recover their sensitivity to growth factor stimulation.

The temporal restriction of negative feedback mechanisms is critical for many developmental processes in which cells need to respond to the same ligand multiple times in order to become committed to a certain fate. The enhanced degradation of Sprouty in response to the same signaling events that set its expression suggests a mechanism whereby the repressive activity of a negative feedback loop can be limited to a defined period following receptor activation.

Supplementary Material

Supplementary Material including the Experimental Procedures as well as one supplementary figure is available with this article online at http://images.cellpress.com/supmat/supmatin.htm.

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