

# Spred2 interaction with the late endosomal protein NBR1 down-regulates fibroblast growth factor receptor signaling

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The potential for modulation of growth factor signaling by endocytic trafficking of receptors is well recognized, but the underlying mechanisms are poorly understood. We examined the regulation of fibroblast growth factor (FGF) signaling by Sprouty related with EVH1 (Ena/VASP homology 1) domain (Spred), a family of signaling inhibitors with proposed tumor-suppressive functions. The inhibitory activity of Spreds has been linked to their N-terminal EVH1 domain, but the molecular mechanism is unknown. In this study, we identify a novel late

endosomal protein that directly binds to the EVH1 domain of Spred2. Neighbor of BRCA1 (NBR1) is a highly conserved multidomain protein that interacts and colocalizes with Spred2 in vivo. Attenuation of FGF signaling by Spred2 is dependent on the interaction with NBR1 and is achieved by redirecting the trafficking of activated receptors to the lysosomal degradation pathway. Our findings suggest a critical function for NBR1 in the regulation of receptor trafficking and provide a mechanism for down-regulation of signaling by Spred2 via NBR1.

## Introduction

Growth factors regulate key aspects of cellular life such as proliferation, differentiation, migration, and death. Pattern formation and organogenesis during development, along with tissue regeneration and repair during adulthood, are dependent on strictly regulated action of growth factors. However, deregulated activity of these factors or their downstream signaling pathways can result in developmental disorders as well as contribute to a wide variety of cancers. In fact, loss of function mutations of growth factor signaling antagonists or gain of function mutations of growth factor signaling agonists are a hallmark of many tumors (Vogelstein and Kinzler, 2004).

Precise regulation of growth factor signaling is achieved by a large body of extrinsic and intrinsic regulators of signaling, the majority of which remain poorly defined. Sprouty related

with EVH1 domain (Spred) and its related Sprouty proteins are two such families of intrinsic signaling regulators that inhibit the RAF–MEK (MAPK/extracellular signal-regulated kinase [ERK])–ERK (ERK1/2) pathway downstream of a variety of stimuli (Hacohen et al., 1998; Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999; Wakioka et al., 2001; Kato et al., 2003; Nonami et al., 2004; Bundschu et al., 2005; King et al., 2005; Sivak et al., 2005). Sprouty was initially discovered in *Drosophila melanogaster* as a negative regulator of Bnl (Branchless) FGF signaling during development of the tracheal system (Hacohen et al., 1998) but was subsequently shown to attenuate signaling from other growth factors of the receptor Tyr kinase (RTK) family as well, establishing it as a general RTK antagonist (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999). However, vertebrate Sproutys were shown to inhibit ERK1/2 downstream of only a subset of RTK growth factors such as FGF and VEGF but not EGF (Minowada et al., 1999; Impagnatiello et al., 2001).

All Sprouty proteins share a characteristic Cys-rich C-terminal domain (SPRY domain), which is believed to be indispensable for their function (Casci et al., 1999; Yigzaw et al.,

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Abbreviations used in this paper: BafA, bafilomycin A1; ERK, extracellular signal-regulated kinase; EVH1, Ena/VASP homology 1; FGFR, FGF receptor; IB, immunoblot; IP, immunoprecipitation; KBD, Kit-binding domain; MPR, mannose 6-phosphate receptor; NBR1, neighbor of BRCA1; RTK, receptor Tyr kinase; Spred, Sprouty related with EVH1 domain; Tet, tetracycline; TR, Tet-repressor; UBA, ubiquitin associated; WCL, whole cell lysate; WT, wild type.

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2001; Hanafusa et al., 2002). Spreds also contain a C-terminal SPRY domain but diverge from Sproutys by further containing a central Kit-binding domain (KBD) and an N-terminal EVH1 (Ena/VASP homology 1) domain (Wakioka et al., 2001). Moreover, Spreds are divergent with regard to their target stimuli, inhibiting ERK1/2 downstream of a diverse group of RTK and non-RTK factors such as FGF, EGF, cytokines, and chemokines (Wakioka et al., 2001; Kato et al., 2003; Nonami et al., 2004; Bundschu et al., 2005; King et al., 2005; Sivak et al., 2005).

Similar to Sproutys, Spreds are thought to be potential tumor suppressors, as expression of both Spred1 and -2 has been shown to be reduced in human hepatocellular carcinomas, with levels negatively correlating with malignancy (Yoshida et al., 2006). Apart from their role as potential tumor suppressors, knockout studies have highlighted the involvement of Spreds in bone morphogenesis (Bundschu et al., 2005), hematopoiesis (Nobuhisa et al., 2004), allergen-induced airway eosinophilia, and hyperresponsiveness (Inoue et al., 2005). Furthermore, Spreds have been implicated along with Sproutys in the regulation of *Xenopus laevis* gastrulation and mesoderm formation downstream of FGF, although both the timing and the target pathway of Spreds seemed to be distinct from that of Sproutys (Sivak et al., 2005).

Previous studies have shown that the N-terminal EVH1 domain of Spreds is essential for their inhibitory activity on ERK1/2 (Wakioka et al., 2001; King et al., 2005). However, the molecular mechanism of this EVH1-dependent action is unknown. Because EVH1 domains are protein–protein interaction modules (Ball et al., 2002), we hypothesized that an unidentified critical partner of Spreds might interact with the EVH1 domain to mediate their function. Therefore, we used a yeast two-hybrid approach, using the EVH1 domain of Spred2 as bait to identify candidate partners. We identified neighbor of BRCA1 (NBR1), a multidomain protein which contains several putative protein–protein interaction modules, an N-terminal homo/heterodimerization PB1 (Phox and Bem1p1) domain, a ZINC finger, a coiled-coil region, and a C-terminal ubiquitin-associated (UBA) domain. NBR1 has been previously implicated in signal transduction downstream of the giant muscle kinase Titin (Lange et al., 2005), but its roles outside the muscle context are otherwise poorly characterized. In this study, we show that NBR1 is a specific late endosomal protein. Spred2 interacts and colocalizes with NBR1 in an EVH1 domain–dependent manner in vivo. Moreover, inhibition of ERK1/2 by Spred2 is dependent on its interaction with NBR1 and is achieved by targeting activated receptors to the lysosomal degradation pathway. Our findings provide a mechanism for the EVH1-dependent actions of Spred2 and implicate NBR1 as a novel regulator of receptor trafficking and signaling for the first time. These results further support the notion that alteration of receptor trafficking itinerary could be a common means for regulation of downstream signaling.

## Results

### NBR1 is a novel EVH1 domain binding partner of Spred2

Using the EVH1 domain of murine Spred2 (mSpred2), which has 100% homology with its human counterpart, as bait in a

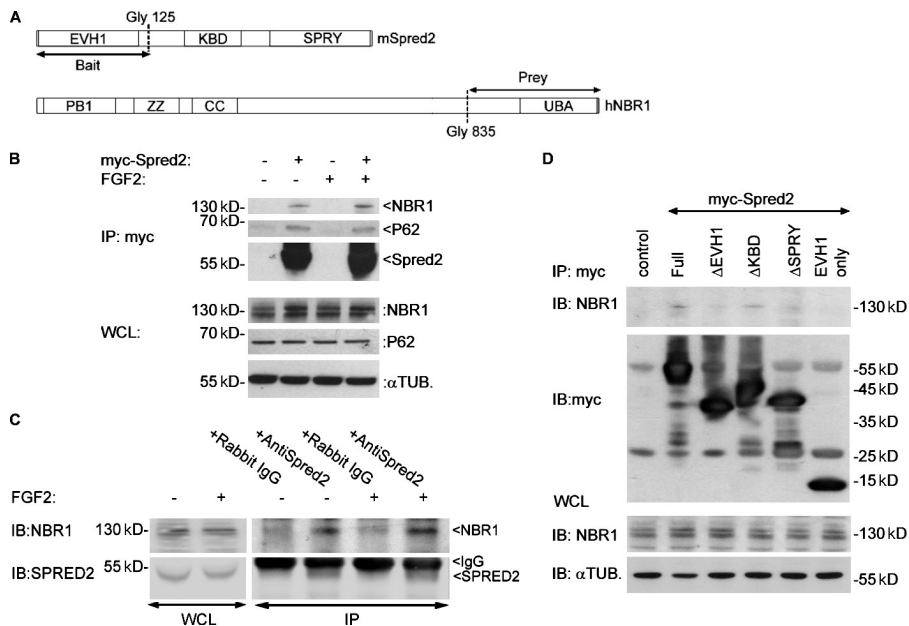
yeast two-hybrid screen of a human skeletal muscle cDNA library ( $\sim 5 \times 10^5$  colonies), we identified NBR1 as a novel binding partner. Of the total 22 positive hits, >70% corresponded to the C-terminal end of NBR1. The shortest cDNA encoded for the 131 C-terminal amino acids of the protein, suggesting that the binding site for the Spred2 EVH1 domain is located within these 131 amino acids (Fig. 1 A). The existence of a direct interaction between this region of NBR1 and the EVH1 domain of Spred2 was further confirmed by a directed yeast two-hybrid assay (Fig. S1 A) as well as an in vitro pull-down experiment (Fig. S1 B). It should be noted that there are no canonical Pro-rich EVH1-binding sequences within this region of NBR1 in accord with the previously solved tertiary structure of the Spred EVH1 domain, which indicates a distinct binding mechanism to other EVH1 domains (Harmer et al., 2005).

We then performed coimmunoprecipitation (co-IP) experiments in mammalian cells to confirm the existence of an interaction in vivo. GFP-tagged NBR1 could be immunoprecipitated with myc-Spred2 in human embryonic kidney 293T cells (Fig. S1 C). Endogenous NBR1 could similarly be immunoprecipitated with myc-Spred2 in 293T cells (Fig. 1 B). We also found in these experiments that endogenous P62, another PB1 domain– and UBA domain–containing protein which is known to interact with NBR1 (Lange et al., 2005), also coimmunoprecipitated with myc-Spred2 (Fig. 1 B). Interestingly, although endogenous NBR1 appeared as a doublet in 293T cell lysates, only the higher migrating band seemed to be immunoprecipitated with Spred2 (Fig. 1 B).

To confirm the interaction of NBR1 with SPRED2 in a physiological context, we used a neuronal cell line (SH-SY5Y neuroblastoma), as SPRED2 has been shown to be expressed at relatively high levels in neuronal tissues (Engelhardt et al., 2004; Bundschu et al., 2006). Endogenous NBR1 could be immunoprecipitated with endogenous SPRED2 in these cells (Fig. 1 C), supporting the existence of a Spred–NBR1 interaction at physiological protein levels. Finally, as predicted from the yeast two-hybrid assay, the interaction of Spred2 with endogenous NBR1 in vivo was dependent on the EVH1 domain (Fig. 1 D). Surprisingly, the SPRY domain was also necessary for an interaction in vivo, whereas KBD was dispensable (Fig. 1 D).

### Spred2 colocalizes with NBR1 in an EVH1 domain–dependent manner

Next, we investigated the subcellular localization of Spred2 and NBR1 by confocal microscopy. In COS-7 cells, we found that the majority of ectopically expressed Spred2 exhibited a cytoplasmic punctuate staining (Fig. 2 A). Similar staining was observed for endogenous SPRED2 in SH-SY5Y neuroblastoma cells (Fig. 2 B), ruling out the possibility of an artifact caused by overexpression. To investigate the subcellular localization of NBR1, we transfected COS-7 cells with a C-terminally GFP-tagged NBR1 construct. NBR1-GFP was found to localize to the limiting membranes of some perinuclear vesicular structures (Fig. 2 C). These vesicles were occasionally found attached to one another, which gave them an



**Figure 1. NBR1 is a novel Spred2 binding partner.** (A) A schematic representation of Spred2 and NBR1 domains. The N-terminal EVH1 domain of Spred2 was used as bait in a yeast two-hybrid screen that identified NBR1. The shortest cDNA of NBR1 identified coded for the 131 C-terminal amino acids. CC, coiled-coil; hNBR1, human NBR1; ZZ, ZZ-type ZINC finger domain. (B) Endogenous NBR1 as well as its interacting protein P62 can be immunoprecipitated with myc-tagged Spred2 from 293T cell lysates, irrespective of FGF2 stimulation. IP and WCL samples were immunoblotted with the indicated antibodies (IB). (C) Endogenous NBR1 can also be immunoprecipitated with endogenous Spred2 from SH-SY5Y neuroblastoma cell lysates using an anti-Spred2 antibody, irrespective of FGF2 stimulation. Approximately 4 mg of cell lysate was used for each IP along with 15 µg of either anti-Spred2 or nonspecific rabbit IgG antibodies. (D) Both the EVH1 and SPRY domains of Spred2 are necessary for the interaction with NBR1 in vivo. Endogenous NBR1 can only be immunoprecipitated from 293T cell lysates with either wild type (WT) or the KBD-deleted mutant of Spred2 but not the EVH1 domain- or SPRY domain-deleted mutants. Note that in all sections, film exposure times for the NBR1 WCL blots were significantly more than for the NBR1 IP blots. TUB, tubulin.

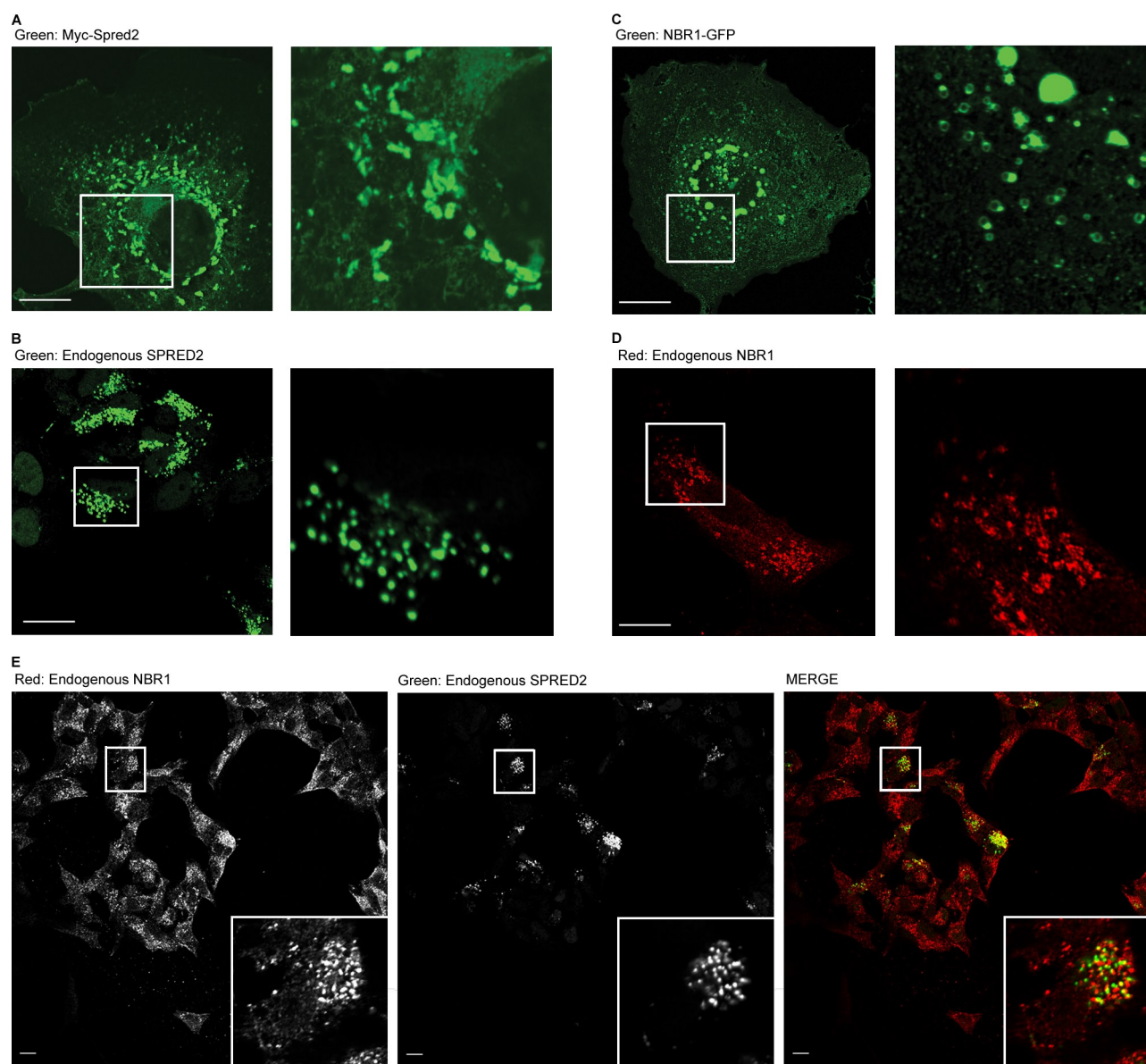
aggregate-like appearance, but this was dependent on the level of NBR1-GFP expression. The same vesicular localization was observed with an N-terminally GFP-tagged mNbr1 (unpublished data), suggesting that it must be a conserved feature and that it is not affected by the position of the tag. Endogenous levels of NBR1 were below detection limits by immunofluorescence in all cells that we investigated. However, while this manuscript was being revised, two other papers were published showing that by treating the cells with the lysosomal inhibitor bafilomycin A1 (BafA), endogenous NBR1 levels could be increased to immunofluorescence-detectable levels, as NBR1 is degraded via the lysosomal degradation pathway (Kirkin et al., 2009; Waters et al., 2009). This was in fact the case, and we could detect endogenous NBR1 in COS-7 cells treated with BafA (Fig. 2 D). Similar to GFP-tagged NBR1, endogenous NBR1 also exhibited a vesicular localization in these cells (Fig. 2 D).

We next investigated whether NBR1 and SPRED2 colocalize in vivo. In BafA-treated SH-SY5Y cells, endogenous NBR1 and SPRED2 colocalized in cytoplasmic punctae (Fig. 2 E). Similarly, ectopically expressing Spred2 and NBR1-GFP were found to colocalize in vivo (Fig. 3 A). In agreement with the IP results (Fig. 1 D), deletion of either the EVH1 or SPRY domain abolished this colocalization, whereas deletion of KBD had no effect (Fig. 3, B–D). Interestingly, deletion of the SPRY domain had a profound effect on Spred2 localization, causing it to entirely lose its punctate cytoplasmic localization and mainly mislocalize to the nucleus (Fig. 3 D). This finding can explain why both the EVH1 and SPRY domains of Spred2 are found to be required for an interaction in vivo (Fig. 1 D): EVH1 is required for protein–protein interaction, and SPRY is required for vesicular localization.

### NBR1 specifically localizes to the limiting membrane of late endosomes

We next set out to characterize the vesicular structures to which NBR1 was localized. No colocalization was observed between NBR1-GFP and the early endosomal marker EEA1 (Fig. 4 A) or the recycling endosomal marker RAB11 (Fig. 4 B). The same was also observed with another early endocytic marker, RAB5 (Fig. S2 A). However, a very strong colocalization was detected between NBR1 and the late endosomal/lysosomal marker LAMP2 (Fig. 4 C) or RAB7 (Fig. S2 B). To discriminate between late endosomes and lysosomes, we used an antibody against mannose 6-phosphate receptor (MPR), a late endosomal marker which is specifically absent in lysosomes. A strong colocalization between NBR1-GFP and MPR was detected (Fig. 4 D). Conversely, when LysoTracker red was used, an acidotrophic dye which specifically stains lysosomes, no colocalization with NBR1-GFP was observed (Fig. 4 E). Similarly, no colocalization was observed between LysoTracker red and myc-tagged Nbr1, ruling out the possibility of GFP inactivation as the result of low lysosomal pH (Fig. S2 C). This was while myc-Nbr1 and LAMP2 still perfectly colocalized (unpublished data). Late endosomal localization of NBR1 could also be shown for endogenous NBR1 in cells treated with BafA (Fig. 4 F). Finally, no colocalization was also observed between NBR1-GFP and the caveosome marker CAV1 (Fig. S2 D) or NBR1-GFP and GM130, a specific Golgi marker (Fig. S2 E). These results collectively show that NBR1-positive vesicles are specifically late endosomal in character. As NBR1 gets degraded in lysosomes (Kirkin et al., 2009), we therefore believe that its specific late endosomal localization is a steady-state phenomenon, being continuously trafficked from late endosomes to lysosomes but then getting rapidly degraded and therefore





**Figure 2. Endogenous NBR1 and Spred colocalize with each other.** (A) The majority of myc-Spred2 in COS-7 cells exhibits a punctate cytoplasmic staining. (B) Endogenous SPRED2 also localizes to cytoplasmic punctae in SH-SY5Y neuroblastoma cells. (C) GFP-tagged NBR1 is localized to the limiting membrane of some vesicular structure in COS-7 cells. (D) Endogenous NBR1 similarly localizes to vesicular structures in COS-7 cells treated with BafA. (E) Endogenous NBR1 colocalizes with endogenous SPRED2 in SH-SY5Y cells treated with BafA. (D and E) Cells were subjected to 200 nM BafA for 16 h before analysis. (A–E) Images on the right (A–D) or insets (E) are higher magnifications of the boxed areas. Bars, 10  $\mu$ m.

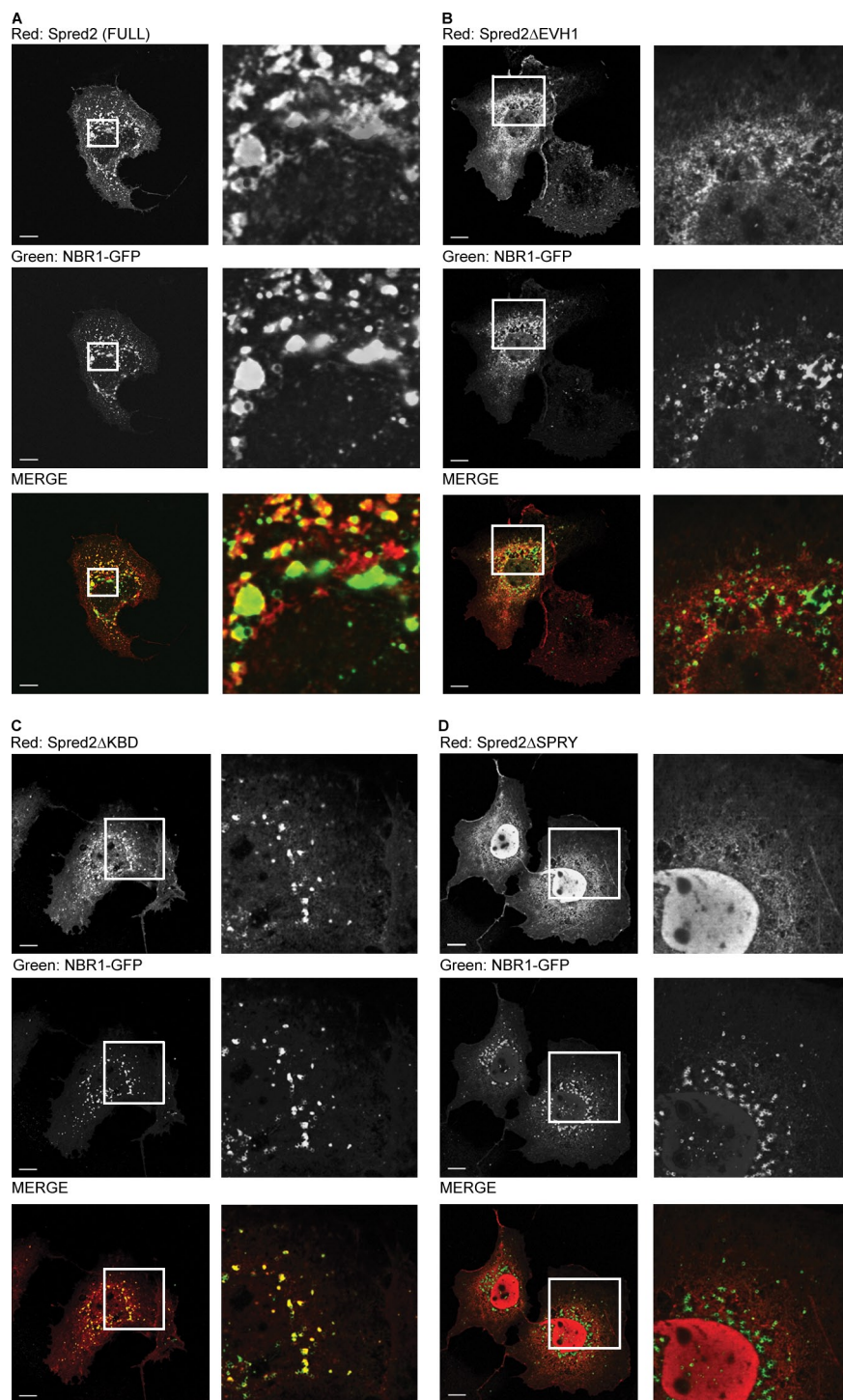
not detected in significant levels within the lysosomal compartment of our cells.

Late endocytic localization of NBR1 is interesting in the view that NBR1 partner P62 has also been previously shown to localize to the late endosomal/lysosomal compartment (Sanchez et al., 1998). In fact, we could show that a strong colocalization existed between P62 and NBR1 when both proteins were expressed in COS-7 cells (Fig. S3 A). When Spred2 was also expressed, it colocalized with NBR1–P62-positive endosomes (Fig. S3 B), which is in agreement with the earlier co-IP results (Fig. 1 B). Furthermore, when lysates of 293T cells ectopically expressing NBR1, P62, and Spred2 were subjected to blue native/SDS two-dimensional gel electrophoresis, a fraction of these proteins co-migrated in the native

dimension as a single high molecular mass multiprotein complex that could be disrupted with pretreatment of the lysate with 1% SDS (Fig. S3 C). Therefore, we conclude that a NBR1–P62–SPRED multiprotein complex might exist in the late endosomal compartment in vivo.

#### ERK1/2 inhibition by Spred2 is dependent on the interaction with NBR1

We next investigated the functional significance of the interaction with NBR1 for Spred2-mediated inhibition of ERK1/2. We observed that expression of myc-tagged Spred2 in 293T cells reduced FGF2-mediated ERK1/2 activity at various time points after stimulation (Fig. 5 A and Fig. S4 A). In agreement with previous studies (Wakioka et al., 2001; King et al., 2005), this



**Figure 3. Colocalization of Spred2 with NBR1 is dependent on both the EVH1 and SPRY domains.** (A) NBR1-GFP colocalizes with WT myc-Spred2 in COS-7 cells. (B) NBR1-GFP does not colocalize with EVH1-deleted myc-Spred2. (C) NBR1-GFP colocalization with myc-Spred2 is not affected by deletion of the KBD. (D) NBR1-GFP colocalization with myc-Spred2 also depends on the presence of the SPRY domain, probably because of the mislocalization of SPRY-deleted Spred2 to the nucleus. (A–D) Images on the right are higher magnifications of the boxed areas on the left. Bars, 10  $\mu$ m.

inhibition of ERK1/2 activity by Spred2 was dependent on both EVH1 and SPRY domains (Fig. 5 A), the two domains which were also essential for interaction and colocalization with NBR1 (Figs. 1 D and 3, B and D). In contrast, KBD, which was shown to be dispensable for interaction and colocalization with NBR1 (Figs. 1 D and 3 C), did not affect Spred2-mediated inhibition of ERK1/2 (Fig. 5 A). Therefore, ERK1/2 inhibition by Spred2 mutants correlates with their ability to interact with NBR1.

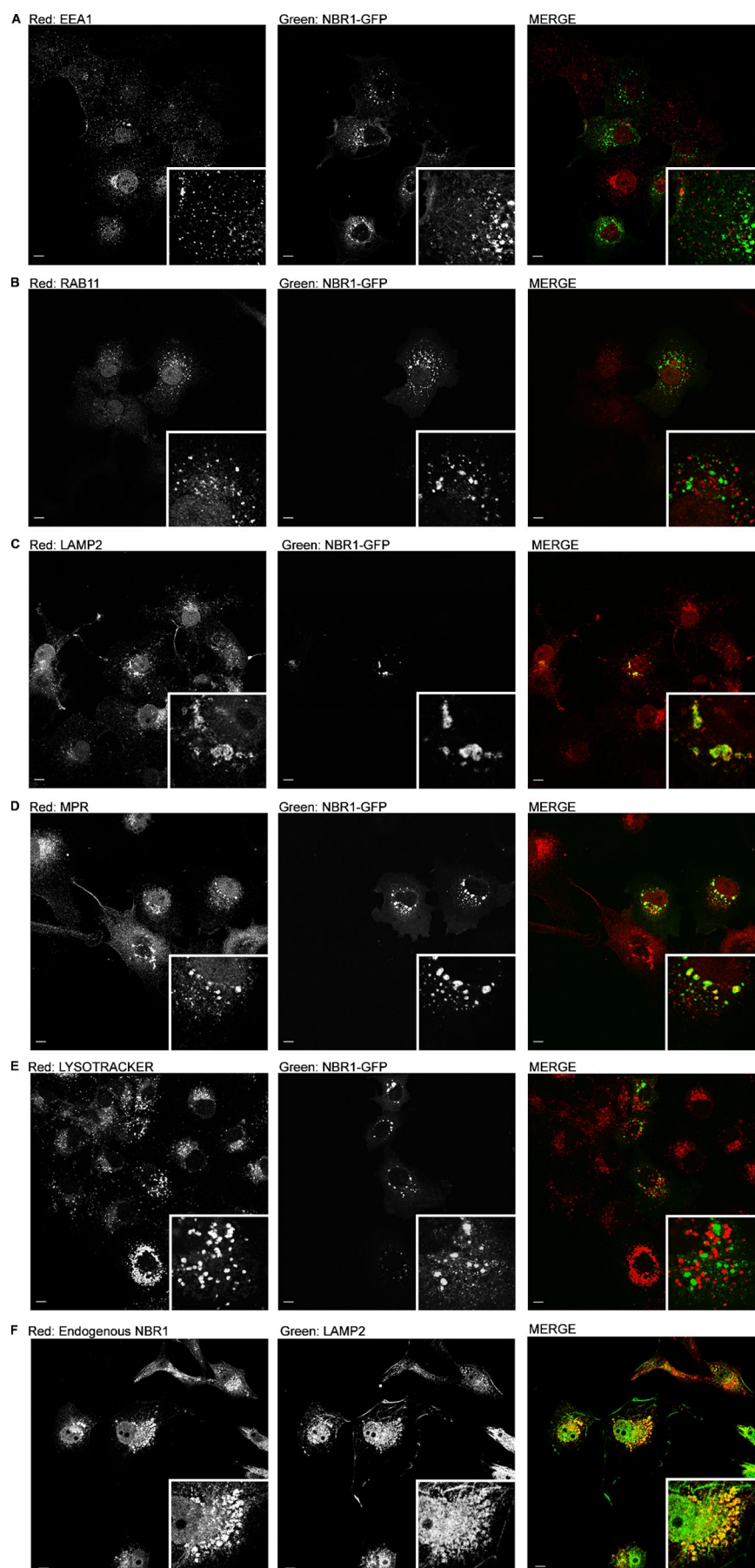
We then investigated whether NBR1 could cooperate with Spred2 in inhibiting ERK1/2 activity. When expressed on

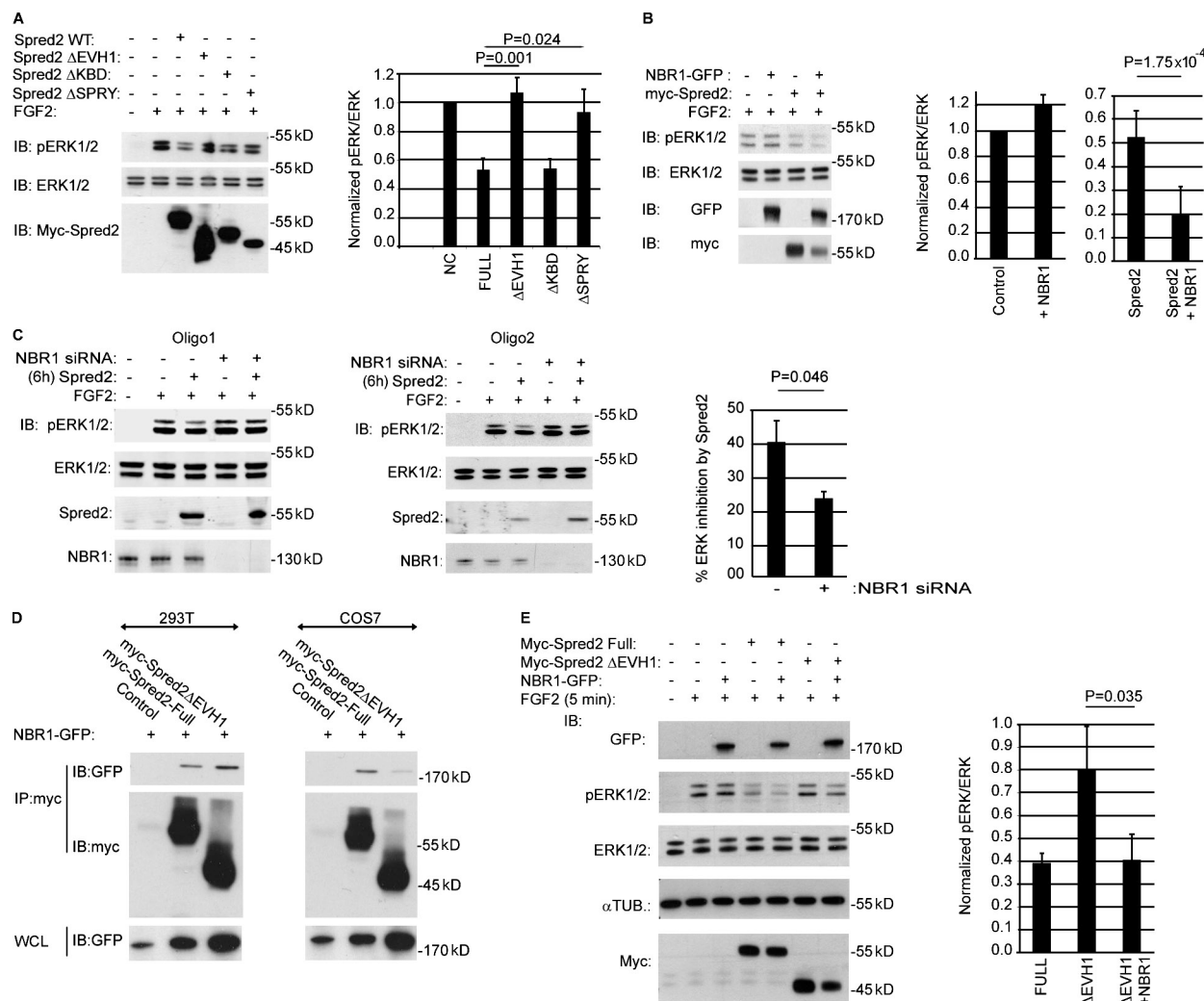
its own, NBR1 did not reduce ERK1/2 activity (Fig. 5 B). However, when coexpressed along with Spred2, NBR1 further enhanced Spred2-mediated ERK1/2 inhibition (Fig. 5 B). This enhancement of Spred2 activity supports the notion that the formation of a Spred2–NBR1 complex is important for Spred2-mediated inhibition of ERK1/2, as coexpression of NBR1 must be forcing more of Spred2 into forming a complex with NBR1.

Subsequently, we assessed the effect of endogenous NBR1 depletion by siRNA on Spred2 function. We used a tetracycline



**Figure 4. NBR1 is specifically localized to the limiting membrane of late endosomes.** (A) No colocalization between NBR1-GFP and endogenous EEA1, the marker of early endosomes, is seen in COS-7 cells. (B) No colocalization is also observed between NBR1-GFP and RAB11, the marker of recycling endosomes. (C) A strong colocalization is detected between the late endosomal/lysosomal marker LAMP2 and NBR1-GFP. (D and E) NBR1-GFP also colocalizes strongly with the specific late endosomal marker MPR (D) but not with lysosomes that stain positive for LysoTracker red (E). (F) Endogenous NBR1 also colocalizes with the late endosomal/lysosomal marker LAMP2 in COS-7 cells treated with BafA. Cells were subjected to 200 nM BafA for 16 h before analysis. (A–F) Insets are higher magnifications of their associated pictures. Bars, 10  $\mu$ m.

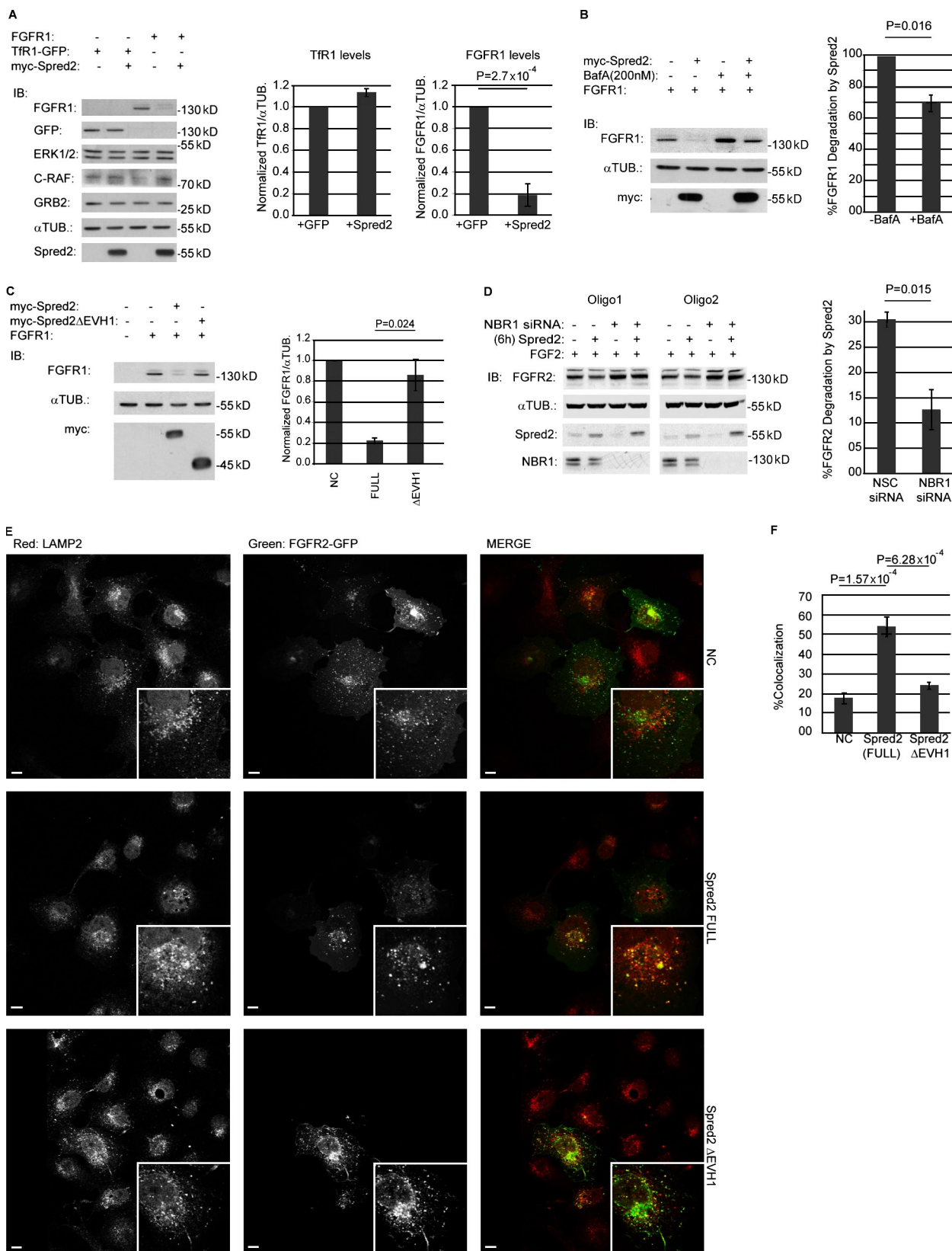




**Figure 5. Inhibition of ERK1/2 activity by Spred2 is dependent on the interaction with NBR1.** (A, left) 293T cells stimulated with FGF2 in the presence of WT or various deletion mutants of myc-Spred2 were analyzed by IB for ERK1/2 activity. Inhibition of ERK1/2 by myc-Spred2 was significantly reduced by the loss of both the EVH1 and SPRY domains but not KBD. (right) Densitometric analysis of pERK1/2 levels from left. The p-values were calculated using one-tailed nonpaired *t* tests ( $n = 6$ ). NC, negative control. (B) NBR1 overexpression enhances Spred2-mediated ERK1/2 inhibition. (left) GFP or NBR1-GFP plus either empty vector- or myc-Spred2-cotransfected 293T cells were FGF2 stimulated. NBR1-GFP on its own did not affect pERK1/2 levels, but when coexpressed with myc-Spred2, it significantly enhanced the ability of Spred2 to reduce pERK1/2 levels. Threefold more NBR1-GFP (or GFP for controls) was transfected to ensure that the majority of myc-Spred2-expressing cells also expressed NBR1-GFP. (right) Densitometric analysis of the pERK1/2 levels from the left. The p-value was calculated using a one-tailed paired *t* test ( $n = 3$ ). (C) Depletion of endogenous NBR1 impairs Spred2 activity. (left) 293T cells were double transfected (with 100 pmol and then 50 pmol per 10-cm well) with either nonsilencing control or NBR1-specific siRNA oligonucleotides. The Tet-inducible Spred2 construct was cotransfected with TR (1:6 ratio to ensure that every Spred-transfected cell also expressed TR) along with the oligonucleotides on the second transfection. Spred2 expression was induced 6 h before stimulation by 1  $\mu$ g/ml Tet. Two independent NBR1 siRNA oligonucleotides were used (Oligo1 and -2). Spred2 inhibition of ERK1/2 activity was significantly reduced in both cases. (right) Densitometric analysis of the left. The p-value was calculated using a one-tailed paired *t* test ( $n = 5$ ). (D) Ectopic expression of NBR1 can rescue NBR1 interaction with the EVH1-deleted Spred2 mutant in a cell type-specific manner. 293T or COS-7 cells overexpressing NBR1-GFP along with WT myc-Spred2,  $\Delta$ EVH1 myc-Spred2, or empty vector as negative control were subjected to IP by myc antibody. Although, like endogenous NBR1, the interaction of ectopic NBR1 with Spred2 was EVH1 domain dependent in COS-7 cells, ectopic NBR1 could still interact with  $\Delta$ EVH1 Spred2 in 293T cells. Note that the film exposure time for the NBR1 WCL blot was significantly more than for the NBR1 IP blot. (E) Rescuing the NBR1-Spred2 interaction also rescues the function of a noninteracting, otherwise nonfunctional  $\Delta$ EVH1 Spred2 mutant. (left) 293T cells expressing either GFP or NBR1-GFP plus WT myc-Spred2,  $\Delta$ EVH1 myc-Spred2, or empty vector as negative control were stimulated with FGF2 as shown.  $\Delta$ EVH1 myc-Spred2 could not reduce ERK1/2 activity alone, but pERK1/2 reduction by the  $\Delta$ EVH1 myc-Spred2 mutant was rescued back to WT levels when ectopic NBR1-GFP was also expressed to rescue the interaction. NBR1-GFP expression on its own did not reduce ERK1/2 activity. Threefold more NBR1-GFP (or GFP for controls) was transfected to ensure that the majority of Spred2-expressing cells also expressed NBR1-GFP. (right) Densitometric analysis of the left. The p-value was calculated using a one-tailed paired *t* test ( $n = 3$ ). TUB, tubulin. (A–C and E) Error bars represent SEM.

(Tet)-inducible construct to make sure that Spred2 was expressed only after NBR1 was sufficiently depleted. Although induction of Spred2 in a control siRNA background inhibited ERK1/2 activity as expected (Fig. 5 C), knockdown of NBR1 by specific siRNA oligonucleotides significantly reduced this

inhibition (Fig. 5 C). To rule out the possibility of an off-target effect, two independent siRNA oligonucleotides were used, both of which gave similar results (Fig. 5 C). These results reveal that Spred2-mediated inhibition of ERK1/2 activity is dependent on NBR1. Interestingly, as we tried to improve the



**Figure 6. Spred2 targets activated receptors to the lysosomal degradation pathway in an EVH1 domain-dependent manner.** (A) Spred2 expression results in degradation of ectopic active FGFR1. (left) Levels of ectopic active FGFR1 are significantly decreased in lysates of myc-Spced2- but not GFP control-cotransfected 293T cells, whereas those of ectopic TrfR1-GFP or downstream signaling components such as ERK, C-RAF, or GRB2 do not change. Fivefold more Spred2 (or GFP for controls) was transfected to ensure that the majority of receptor-expressing cells also coexpressed myc-Spced2. (right) Densitometric analysis of the left for TrfR1 and FGFR1. The p-value was calculated using a one-tailed paired *t* test ( $n = 3$ ). (B) Spred2-mediated degradation of FGFR1 is via the lysosomal degradation pathway. (left) Treatment of 293T cells 1 h before transfection with BafA hinders myc-Spced2-mediated FGFR1



NBR1 knockdown efficiency by performing more rounds of siRNA transfection, we found that cell viability was severely compromised (Fig. S4 C). This was caused by induction of apoptosis and a specific NBR1 phenotype, as it was just seen with NBR1 oligonucleotides but not with nontargeting control siRNA oligonucleotides (Fig. S4 C) or oligonucleotides against P62 (not depicted). This suggests the existence of some vital functions for NBR1, which result in cell death when the protein is sufficiently depleted. Given that knockouts of both *Spred1* and *-2* have been generated and are viable (Nobuhisa et al., 2004; Bundschu et al., 2005; Inoue et al., 2005) and because expression of the nonfunctional EVH1-deleted *Spred2* mutant also does not affect cell viability, NBR1 knockdown lethality is very likely independent of *Spred2*. The nature of these vital functions remains to be determined.

We next set out to investigate whether rescue of the *Spred2*–NBR1 interaction would also rescue the function of an EVH1-deleted *Spred2* mutant. While investigating the interaction of NBR1 and *Spred2*, we made a surprising observation. Despite the fact that *Spred2* interaction with endogenous NBR1 in 293T cells required both the EVH1 and SPRY domains (Fig. 1 D), ectopic expression of NBR1 in 293T cells could rescue the interaction with an EVH1-deleted *Spred2* mutant (Fig. 5 D). This was a cell type–specific phenomenon, as it could not be seen in other cell types investigated such as COS-7 cells (Fig. 5 D). The ability to rescue the *Spred2*–NBR1 interaction in a cell type–specific manner for an otherwise noninteracting/nonfunctional  $\Delta$ EVH1 *Spred2* mutant provided us with another way to test the functional significance of this interaction. We hypothesized that if the role of the EVH1 domain is to interact with NBR1, ectopic expression of NBR1 in 293T cells should rescue the inability of the  $\Delta$ EVH1 *Spred2* mutant to inhibit ERK1/2. This was in fact the case. Although expression of the  $\Delta$ EVH1 *Spred2* mutant alone was not capable of inhibiting FGF2-mediated ERK1/2 activity, inhibition of ERK1/2 was restored when NBR1 was coexpressed to rescue the interaction (Fig. 5 E). However, as before, NBR1 expression on its own did not affect ERK1/2 activity (Fig. 5 E). These data strongly suggest that *Spred2*-mediated inhibition of ERK1/2 is dependent on the interaction with NBR1 and that the role of the EVH1 domain is to bind NBR1.

### **Spred2 targets activated receptors for lysosomal degradation in an EVH1 domain-dependent manner**

Finally, we addressed the mechanism by which *Spred2* results in attenuation of signaling. Considering the localization of NBR1 to the late endosomal compartment, we reasoned that *Spred2* might regulate the endocytic trafficking of activated receptors via interaction with NBR1. It is widely recognized that receptor endocytosis can act to down-regulate signaling by targeted degradation of activated receptors via the lysosomal degradation pathway. Therefore, we examined whether *Spred2* acts to enhance degradation of activated receptors. 293T cells were transfected with either FGF receptor 1 (FGFR1) or a tagged transferrin receptor 1 (TrfR1) as a control, with or without *Spred2*, and subjected to analysis by immunoblotting. We did not stimulate cells with FGF, as overexpression of FGFR1 is autoactivating (Ong et al., 2000). A marked decrease in the level of ectopic FGFR1 was seen in the presence of *Spred2*, whereas levels of TrfR1 did not change significantly (Fig. 6 A), ruling out the possibility of a nonspecific effect on cotransfected protein levels by *Spred2*. No decrease in the levels of downstream signaling components GRB2, C-RAF, or ERK was detectable either (Fig. 6 A), indicating that the effect of *Spred2* is restricted to the receptor itself. A steady decrease in FGFR1 receptor levels was also seen in *Spred2*-expressing cells treated for various times with the translational inhibitor cycloheximide (Fig. S4 B). Moreover, *Spred2*-induced reduction in FGFR1 levels could be inhibited by treating cells with BafA, providing evidence for mediation of the decrease via the lysosomal degradation pathway (Fig. 6 B). Another lysosomal inhibitor, chloroquine, also gave similar results (Fig. S4 D). Next, we compared full-length *Spred2* with the  $\Delta$ EVH1 *Spred2* mutant, which lacks the ability to interact with NBR1. Although full-length *Spred2* decreased the level of ectopic FGFR1 as before,  $\Delta$ EVH1 *Spred2* was incapable of inducing such reduction (Fig. 6 C). We also investigated the effect of *Spred2* expression on endogenous FGFR levels and whether this was NBR1 dependent. We monitored endogenous FGFR2, as it was readily detectable in 293T cells. To make sure *Spred2* was only expressed after NBR1 was sufficiently depleted, we used Tet-inducible *Spred2* in the presence of control versus NBR1

degradation. Fivefold more myc-*Spred2* (or GFP for controls) was transfected to ensure that the majority of receptor-expressing cells also coexpressed myc-*Spred2*. Cells were analyzed 12 h after transfection. (right) Densitometric analysis of FGFR1 degradation by myc-*Spred2* in BafA-treated versus untreated cells. The p-value was calculated using a one-tailed paired *t* test (*n* = 3). (C) *Spred2*-induced decrease in FGFR1 levels is EVH1 dependent. (left) Contrary to WT myc-*Spred2*,  $\Delta$ EVH1 myc-*Spred2* does not degrade FGFR1 in cotransfected 293T cells. Fivefold more myc-*Spred2* or  $\Delta$ EVH1 myc-*Spred2* (or GFP for controls) was transfected to ensure that the majority of receptor-expressing cells also coexpressed *Spred2*. (right) Densitometric analysis of the left. The p-value was calculated using a one-tailed paired *t* test (*n* = 3). (D) Depletion of NBR1 impairs *Spred2*-mediated degradation of endogenous FGFR2. (left) 293T cells were double transfected (with 100 pmol and then 50 pmol per 10-cm well) with either nonsilencing control (NSC) or NBR1-specific siRNA oligonucleotides. The Tet-inducible *Spred2* construct was cotransfected with TR (1:6 ratio to ensure that every *Spred2*-transfected cell also expressed TR) along with the oligonucleotides on the second transfection. *Spred2* expression was induced 6 h before stimulation by 1  $\mu$ g/ml Tet. The experiment was performed with two independent NBR1 siRNA oligonucleotides (Oligo1 and -2). *Spred2*-mediated FGFR2 degradation was significantly reduced with both NBR1 siRNA oligonucleotides. (right) Densitometric analysis of the left. The p-value was calculated using a one-tailed paired *t* test (*n* = 4). (E) *Spred2* alters receptor trafficking toward the lysosomal degradation pathway. In COS-7 cells transfected with FGFR2-GFP plus empty vector as control (negative control [NC]), the majority of endosomal receptors do not colocalize with LAMP2. In COS-7 cells transfected with FGFR2-GFP and WT myc-*Spred2*, most of the endosomal receptors colocalize with LAMP2 (+*Spred2* FULL). However, deletion of the EVH1 domain results in the loss of this *Spred2*-induced LAMP2 colocalization (+*Spred2*  $\Delta$ EVH1). To identify *Spred2*-cotransfected cells, coverslips were also stained for *Spred2* (blue) using an anti-*Spred2* antibody (not depicted). Insets are higher magnification of their associated pictures. (F) The percentage of FGFR2-GFP colocalization with LAMP2 as in E was quantified and averaged. Measurements were from nine or more cells for each cotransfection from two separate experiments. The p-value was calculated using a one-tailed nonpaired *t* test (*n* = 9). (A–D and F) Error bars represent SEM. TUB, tubulin. Bars, 10  $\mu$ m.

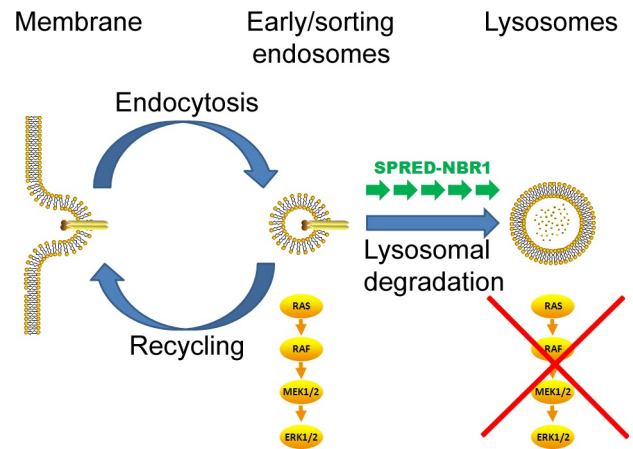
siRNAs as before. In FGF2-stimulated cells, induction of Spred2 expression resulted in a slight reduction of endogenous FGFR2, as expected (Fig. 6 D). However, knockdown of NBR1 by specific siRNA oligonucleotides significantly reduced this effect (Fig. 6 D). As before, two independent siRNA oligonucleotides were used, both of which gave similar results, ruling out the possibility of an off-target effect (Fig. 6 D). Therefore, we conclude that, as with inhibition of ERK1/2 activity, reduced levels of FGFRs by Spred2 appear to be, at least in part, NBR1 dependent.

Finally, we used a functional GFP-tagged FGFR2 (Ahmed et al., 2008) to elucidate the subcellular localization of active FGFR in Spred2- versus empty vector-transfected COS-7 cells. Like FGFR1, overexpression of FGFR2-GFP was autoactivating (unpublished data), so no additional FGF stimulation was necessary. In the absence of Spred2, only a fraction of endosomal FGFR2-GFP was localized to LAMP2-positive endosomes (Fig. 6, E and F). However, in the presence of Spred2, the majority of FGFR2-GFP endosomes were LAMP2 positive (Fig. 6, E and F). This increase in colocalization of the receptor with LAMP2 could also be reversed by deletion of the Spred2 EVH1 domain (Fig. 6, E and F). Together, these results suggest that the EVH1/NBR1-dependent down-regulation of signaling by Spred2 is achieved via directed endosomal trafficking of activated receptors into the late endosomal/lysosomal degradation pathway.

## Discussion

Collectively, the evidence in this study reveals that the EVH1 domain of Spred2 is required to engage with the late endosomal protein NBR1, and this is necessary for Spred2-mediated inhibition of FGF signaling. Our results also suggest that Spred2 functions by diverting receptors into the lysosomal degradation pathway via the interaction with NBR1 (Fig. 7). This adds to the accumulating body of evidence that implicates receptor trafficking as a key hub for regulating signaling dynamics (Vieira et al., 1996; Kranenburg et al., 1999; Sandilands et al., 2007; Sigismund et al., 2008; for review see Polo and Di Fiore, 2006). Several studies have also suggested a role for Sprouty in the regulation of downstream signaling by modulating the endocytic trafficking of active receptors (Wong et al., 2002; Rubin et al., 2003; Kim et al., 2007). However, there seems to be a functional divergence along with a structural divergence between Sprouty and Spred: Sprouty interferes with lysosomal degradation of the receptors by inhibiting Cbl-mediated receptor internalization (Wong et al., 2002; Rubin et al., 2003) and Hrs-dependent early to late endosomal transition (Kim et al., 2007), whereas Spred seems to promote lysosomal targeting of the receptors via engaging with NBR1.

An intriguing issue is the fact that the inhibitory activity of Spred2 seems to be limited to the ERK1/2 pathway (Wakioka et al., 2001; Nonami et al., 2004; King et al., 2005). Several studies have highlighted the fact that endocytosis does not merely act as a means for receptor removal and degradation but that specific endosomal compartments themselves can act as selective platforms for certain downstream signaling



**Figure 7. Schematic representation of the proposed mechanism for Spred.** Via interacting with NBR1, Spred redirects activated receptors to the lysosomal degradation pathway. Down-regulation of downstream signaling can be selective, as those pathways like ERK1/2 that need specific endosomal compartments for efficient activation are affected the most from a shift in the balance between different trafficking routes (e.g., sorting/recycling versus lysosomal).

pathways (for review see Miaczynska et al., 2004). For instance, ERK1/2 activation downstream of the EGF receptor has been shown to be dependent on receptor endocytosis (Vieira et al., 1996; Kranenburg et al., 1999), whereas PLC- $\gamma$  activation occurs on the membrane and does not require internalization of the receptor (Vieira et al., 1996). ERK1/2 activation downstream of NGF in neurons has also been shown to require internalization of the receptor, whereas AKT activation does not (MacInnis and Campenot, 2002). Endosomal localization of certain signaling components and scaffolds is thought to be important for such selective propagation of downstream signals from specific endosomal compartments. For example, the GTP-bound active form of Rap1, a Ras-related small GTPase which can also activate ERK1/2, has been shown to be preferentially enriched on endosomes (Ohba et al., 2003), and Spred2 has also been shown to be capable of inhibiting Rap1 activation (King et al., 2005). Moreover, the scaffolding protein MP1, which acts to selectively activate ERK1 but not other MAPKs such as p38, has been shown to be enriched in late endosomes via the adapter protein p14 (Wunderlich et al., 2001; Teis et al., 2002). Interestingly, in one of the aforementioned studies that came out while this manuscript was under revision, p14 was listed as a potential NBR1-interacting protein identified in a yeast two-hybrid screen (Waters et al., 2009). It remains to be determined whether this interaction occurs *in vivo* and plays a role in Spred2-mediated ERK1/2 inhibition via NBR1. Nevertheless, these considerations predict that in a short timescale, diversion of endosomal receptor trafficking by Spred2 could be preferentially affecting a subset of downstream signaling pathways such as ERK1/2 that require specific endosomal compartments as platforms for signal propagation.

Our findings implicate NBR1 as a novel regulator of receptor trafficking. The means by which NBR1 regulates receptor trafficking are currently unknown, but several lines of evidence point toward an association with polyubiquitin as a

potential mechanism. Ubiquitination plays a central role in the regulation of receptor trafficking, from target recognition and internalization to vesicle sorting and delivery. Key regulators of receptor trafficking such as epsins, Eps15, Hrs, and Tsg10 all coordinate endocytic processes via ubiquitin-binding domains that specifically recognize ubiquitinated targets (Kirkin and Dikic, 2007). NBR1 also has a C-terminal ubiquitin-binding domain (the UBA domain), but the identity of ubiquitinated cargoes that might interact with this domain is unknown at this moment. The two recent studies on NBR1 have suggested that it might have a role in autophagy by targeting specific ubiquitinated cargoes to autophagosomes (Kirkin et al., 2009; Waters et al., 2009). NBR1 was found to directly interact with the autophagosomal protein LC3 and therefore act as an adapter for specific polyubiquitinated cargoes (Kirkin et al., 2009; Waters et al., 2009). Whether there are any links between the autophagic roles of NBR1 and its endocytic trafficking roles uncovered in this study remains to be determined. Alternatively, NBR1 might have a similar but independent ubiquitinated cargo sorting role in the context of endocytic receptor trafficking. In this light, P62, which is similarly known to be involved in autophagy (Pankiv et al., 2007), has been also shown to regulate NGF receptor sorting via regulating TRAF6-dependent K63 polyubiquitination of the receptor (Geetha et al., 2005). Interestingly, in this study, we demonstrated that NBR1, P62, and a fraction of Spred2 colocalize and form a protein complex in vivo (Fig. 1 B and Fig. S3, B and C). Finally, the endosomal adapter protein p14 has also been shown to regulate receptor trafficking (Teis et al., 2006). As mentioned earlier, whether p14 interacts with NBR1 in vivo, and, if it does so, whether regulation of receptor trafficking and signaling by NBR1 depends on this interaction, awaits future investigations.

## Materials and methods

### Plasmid constructs

For yeast two-hybrid screen, the EVH1 domain of mSpred2 (amino acids 1–125) was cloned into pYTH9 at Sall and BglII sites. N-terminal myc-tagged full-length,  $\Delta$ EVH1,  $\Delta$ KBD,  $\Delta$ SPRY, and EVH1-ONLY mSpred2-pRK5 and N-terminal 6 $\times$  His-tagged EVH1-ONLY pDEST17 were made by Gateway cloning (Invitrogen) according to manufacturer's instructions as in Sweet et al. (2008). N-terminal myc-tagged full-length mNbr1-PRK5, N-terminal GFP-tagged mNbr1-pcDNA DEST53, Tet-inducible mNbr1-pcDNA DEST31, and N-terminal GST-tagged C-terminal-ONLY (P856-Y988) mNbr1-pDEST15 vectors were also made by Gateway cloning. The C-terminal GFP-tagged NBR1 construct was a gift from M. Gautel (King's College London, London, England, UK). The N-terminally Flag-tagged P62 construct was a gift from R. Layfield (University of Nottingham, Nottingham, England, UK). C-terminally GFP-tagged TrfR1 was a gift from J. Rappoport (University of Birmingham, Birmingham, England, UK), and C-terminally GFP-tagged FGFR2 was a gift from J. Ladbury (University College London, London, England, UK). The Tet-repressor (TR) expression construct (TR-pcDNA6) was purchased from Invitrogen.

### Reagents and antibodies

Rabbit polyclonal anti-Spred2 and mouse monoclonal anti-Flag (clone M2) and anti- $\alpha$ -tubulin (clone DM 1A) as well as nonspecific rabbit IgG were all purchased from Sigma-Aldrich. P62 (clone 2C11) and NBR1 (clone 6B11) mouse monoclonal antibodies were purchased from Abnova. Mouse monoclonal anti-GFP (clone 3E1) and anti-c-myc (clone 9E10) antibodies were obtained from Cancer Research UK monoclonal antibody services. Rabbit polyclonal anti-FGFR1, -FGFR2, -Rab5, -Rab7,

and -ERK1 and mouse monoclonal anti-phospho-ERK1/2 (pERK1/2; clone E-4) antibodies were all purchased from Santa Cruz Biotechnology, Inc. Mouse monoclonal antibodies against LAMP2 (clone H4B4) and MPR (clone 2G11) and rabbit polyclonal antibody against LAMP2 were purchased from Abcam. Anti-GRB2, -c-Raf, -Cav1, -EEA1, and -GM130 rabbit polyclonal antibodies were purchased from Cell Signaling Technology. Anti-Rab11 antibody was purchased from Invitrogen. LysoTracker red DND-99 and all fluorescently labeled (Cascade blue, Alexa Fluor 488 [green], Alexa Fluor 594 [red], and Texas red) secondary antibodies were also purchased from Invitrogen. Nontargeting control siRNA and NBR1 siRNA oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. and Thermo Fisher Scientific.

### Cell culture, transfection, stimulation, and IP

Cell culture, transfections, and cell lysis were performed as in Sweet et al. (2008). siRNA transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For multiple siRNA transfections, cells were seeded at 20%, and the first transfection was performed immediately followed by second and third transfections at 24-h intervals. For growth factor stimulation, cells were serum starved in serum-free DME for 6 h, and FGF2 to the final concentration of 20 ng/ml was added at various time points along with 10  $\mu$ g/ml heparin (Sigma-Aldrich). IP was performed by the addition of antibody and Sepharose-G (Sigma-Aldrich) beads at the same time to the lysate followed by 1-h to overnight incubation at 4°C. Unless stated otherwise, 200–600  $\mu$ g of whole cell lysate (WCL) was used. Beads were subsequently washed five times with 20 $\times$  beads bed volume of lysis buffer and resuspended in 2 $\times$  SDS-PAGE sample buffer before analysis by immunoblotting. Half of IP and 5–15  $\mu$ g of WCL were usually loaded for immunoblots (IBs).

### Immunofluorescence and confocal microscopy

For immunofluorescence, cells grown on coverslips were fixed with 4% paraformaldehyde-PBS, permeabilized with 0.2% Triton X-100-PBS before being blocked with 4% BSA-PBS, and subjected to primary and fluorescently labeled secondary antibodies in 4% BSA-PBS. For LysoTracker red staining, a 100-nM solution of the dye in culture medium was applied to the cells before fixation for 2 h. Coverslips were mounted on Mowiol solution and analyzed by laser-scanning confocal microscopy. Sequential single section images were taken by a confocal microscope system (TCS SP2; Leica) using a 63 $\times$  objective lens under low speed (200 Hz), minimum pinhole size (20  $\mu$ m), and high EXPAND (6) settings to maximize image resolution. Final images were generated from averaging eight consecutive scans using the Leica confocal software. All further image processing (level adjustments, brightness and contrast settings, and overlaying) was performed by Photoshop 7.0 (Adobe) or ImageJ 1.37 software (National Institutes of Health). For quantification of colocalizations, the ImageJ JACoP plug-in (Bolte and Cordelières, 2006) was used to calculate the percentage of colocalization from Manders' overlapping coefficients (fraction of green overlapping red).

### Online supplemental material

Fig. S1 shows the interaction of Spred2 and NBR1 by directed yeast two-hybrid, in vitro pull-down, and IP. Fig. S2 shows the colocalization of different endocytic markers with NBR1. Fig. S3 shows the colocalization of NBR1, P62, and Spred2 and their co-migration in native gel as a single complex. Fig. S4 shows the regulation of ERK signaling, cell viability, and FGFR1 degradation by Spred2 and NBR1. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200905118/DC1>.

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