To evaluate tyrosine phosphorylation of IκBα after treatment of cells with EPO, lysates were immunoprecipitated with anti-IκBαs and then probed with anti-phosphoryrysin antibodies. To detect serine phosphorylation of IκBs, lysates were immunoblotted without immunoprecipitation and probed directly with antibodies specific for phosphorylation of IκBα serine residues 32 and 36 (Santa Cruz Biotechnology). For Jak2 in vitro kinase assays, after cell lysis Jak2 was immunoprecipitated with 20 μg of a polyclonal antibody (Santa Cruz Biotechnology). Jak2 was then resuspended in kinase buffer and incubated with 5 μg of His6-tagged IκBαs and 1 μg of GST-IκBα (5 μM dihydrothreitol) plus 10 μM ATP and 30 μg full-length recombinant IκBα. After reaction for 30 min, anti-IκBα immunocomplexes were resolved by SDS–PAGE, probed with anti-phosphoryrysin antibodies, and visualized by ECL (Amersham).

Electrophoretic mobility-shift assays (EMSA)

Nuclear extracts were obtained from cerebrocortical cultures. Binding of NF-κB to DNA was assayed with a double-stranded probe labelled with 32P-dUTP that binds to the consensus sequence (Santa Cruz Biotechnology). Nuclear lysates were incubated with the labelled probe for 2 h at 37 °C, resolved on a 7% native polyacrylamide gel, and exposed to X-ray film. Mutated probe was used as a control. Antibodies specific for p50 and p65 NF-κB subunits were used for supershift analysis. In non-neuronal cells, S-nitrosylation (transfer of NO-related species to a critical thiol from 5-nitrosocysteine or other donors) has been reported to block DNA binding by NF-κB. However, this phenomenon did not affect the EMSA results reported here in neurons because S-nitrosocysteine did not prevent EPO-induced binding.

Reporter gene assays

Cerebrocortical cells were transfected with pNFκB-Luc using calcium phosphate precipitation (Stratagene). Two days later, cells were lysed and mixed with luciferase assay reagent (Promega), and the activity was measured in a luminometer. All measurements were normalized against a non-EPO-dependent control plasmid, pCI/CS-UK (Stratagene). Results represent mean of three experiments measured in triplicate.

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Spred is a Sprouty-related-suppressor of Ras signalling

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Cellular proliferation, and differentiation of cells in response to extracellular signals, are controlled by the signal transduction pathways of Ras, Rap and MAP (mitogen-activated protein) kinase. The mechanisms that regulate this pathway are not well known. Here we describe two structurally similar tyrosine kinase substrates, Sprend-1 and Sprend-2. These two proteins contain a cysteine-rich domain related to Sprouty (the SPR domain) at the carboxy terminus. In Drosophila, Sprouty inhibits the signalling by receptors of fibroblast growth factor (FGF) and epidermal growth factor (EGF) by suppressing the MAP kinase pathway. Like Sprouty, Sprend inhibited growth-factor-mediated activation of MAP kinase. The Ras–MAP kinase pathway is essential in the differentiation of neuronal cells and myocytes. Expression of a dominant negative form of Sprend and Sprend-antibody microinjection revealed that endogenous Sprend regulates differentiation in these types of cells. Sprend constitutively associated with Ras but did not prevent activation of Ras or membrane translocation of Rap. Instead, Sprend inhibited the activation of MAP kinase by suppressing phosphorylation and activation of Rap. Sprend may represent a

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Figure 1 Characterization of Spred molecules. a. Amino acid sequence of Spred-1. The EVH-1 domain is shown in blue, the KBD in red, and the SPR domain in green. b. Comparison of the domain structure of murine Spred-1 and -2 with that of murine Sprouty-4 and VASP. c. Tyrosine phosphorylation (PY) of Myc-tagged Spred-1 expressed in 293 cells in response to SCF. IB, immunoblot; IP, immunoprecipitate; TCL, total cell lysates. d. Subcellular localization of Spred. The endogenous Spred-2 protein in PC12 cells was detected using immunofluorescence microscopy without or with antigen peptide. FITC, fluorescein isothiocyanate. The two right panels show a fluorescent microscopic view of wild-type (GFP-WT) and C-terminal truncated (GFP-ΔC) Spred-1 fused to EGFP in PC12 cells.

Figure 2 Effects of Spred on differentiation and Erk2 activity of PC12 cells. a. Wild-type (WT) and N-terminal-ΔN or C-terminal-truncated (ΔC) Spred-1 constructs were expressed in PC12 cells with EGFP, then incubated with 100 ng ml⁻¹ NGF for 3 d. b. PC12 cells transfected with expression vectors encoding wild-type Spred-1 and HA-tagged Erk2 were stimulated with NGF for 30 min. HA-Erk2 was immunoprecipitated and an in vitro kinase assay was performed with MBP as the substrate. IB, immunoblot. c. PC12 cells were microinjected with a mixture of affinity-purified anti-Spred-1 and -2 antibodies, with or without antigen peptide, and then treated with 25 ng ml⁻¹ NGF for 2 d (c) or 5 min (d). Activated Erk was visualized with a monoclonal anti-phosphorylated-Erk antibody and quantified. The y axis shows relative fluorescence intensity of each cell. In d, yellow arrowheads indicate injected cells, and white arrowheads un.injected cells. e, f, Effects of dominant negative Spred. Cells were co-transfected with ΔC-Spred-1 mutant and EGF receptor, then treated with 10 or 50 ng ml⁻¹ EGF for 3 d (e) or 30 min (f).
class of proteins that modulate Ras–Raf interaction and MAP kinase signalling.

From an osteoclast complementary DNA library, we isolated a tyrosine-kinase-binding protein by a yeast two-hybrid system using the c-Kit and c-Fms tyrosine kinase domains as bait. Full-length cDNA of this gene encodes a protein that contains a C-terminal SPR domain and an amino-terminal Ena/Vasodilator-stimulated phosphoprotein (VASP) homology-1 (EH1-1) domain (Fig. 1a). Thus, we named this gene Sprd-1: Sprouty-related protein with EVH1 domain. We found a very similar gene (Sprd-2) in the database and cloned its full-length cDNA (Fig. 1b). Using various deletion mutants, we identified a c-Kit-binding domain (KBD) composed of about 50 amino acids (codon 234–286) (see Supplementary Information Fig. 1). This region is not related to any previously identified tyrosine kinase interaction domains such as SH2, PTB or c-Met-binding domain. Sprd-1 was tyrosine phosphorylated in response to stem cell factor (SCF), platelet-derived growth factor (PDGF) and EGF (Fig. 1c and data not shown), and efficient phosphorylation of Sprd-1 required the KBD region (Supplementary Information Fig. 2). Using immunofluorescence microscopy, we detected localization of endogenous Sprd-2 to the plasma membrane (Fig. 1d). Membrane localization of Sprd was confirmed by exogenously expressed Sprd fused to enhanced green fluorescent protein (EGFP) (Fig. 1d). The C-terminal SPR domain was essential for plasma membrane localization, as a deletion mutant lacking SPR domain (GFP-ΔC) localized in the cytoplasm (Fig. 1d).

Because Sprd contains a Sprouty-related domain, we examined the effect of Sprd on the Ras–MAP kinase pathway. First, we examined the differentiation of PC12 pheochromocytoma cells, which is induced by nerve growth factor (NGF) and dependent on MAP kinase. As shown in Fig. 2a, overexpression of Sprd-1 or Sprd-2 strongly inhibited NGF-induced differentiation of PC12 cells. Both EVH1 and SPR domains were essential for the suppression of differentiation (Fig. 2a, see ΔN and ΔC). Sprd-1 suppressed NGF-induced activation of Erk2 MAP kinase (Fig. 2b). Next, we inhibited endogenous Sprd by microinjecting affinity-purified anti-Sprd-1 and -Sprd-2 antibodies. The microinjection augmented neurite outgrowth of PC12 cells treated with a low concentration of NGF (Fig. 2c) as well as NGF-induced Erk2 activation (Fig. 2d). These results indicate that the endogenous Sprd proteins suppress growth-factor-induced activation of MAP kinase and reduce the threshold of growth factor sensitivity for differentiation in PC12 cells. We noticed that the C-terminal deletion mutant augmented NGF-induced neurite outgrowth of PC12 cells (Fig. 2a), suggesting that the ΔC mutant may function as a dominant negative form against endogenous Sprd proteins. Indeed, overexpression of the ΔC mutant amplified differentiation of PC12 cells treated with a low concentration (10 ng/ml) of EGF (Fig. 2e) and enhanced EGF-induced activation of Erk2 (Fig. 2f).

The negative effects of Sprd on the Ras–MAP kinase pathway were confirmed in a different system. C2C12 cells differentiated into promyocytes when cultured in 2% horse serum (HS) (differentiation medium) for 3–5 days. MAP kinase activity has been shown to drop rapidly after switching to differentiation medium, but recovers after 3 days (Fig. 2f). This decrease in MAP kinase activity in differentiation medium is essential for myotube formation. The levels of Sprd-1 in C2C12 cells had increased by day 2 and dropped sharply on day 3 (Fig. 3a), and were inversely correlated to the levels of MAP kinase activity (Fig. 2f). As shown in Fig. 3b and c, forced expression of dominant negative Ras (N17-Ras) induced morphological changes in C2C12 cells and reduced MAP kinase activity, even under normal growth conditions (20% FBS). Forced expression of wild-type Sprd-1 and -2 exhibited similar effects (Fig. 3b, c). In contrast, C2C12 cells transfected with the constitutively activated form of Raf (ΔN-Raf) or dominant negative mutants (ΔC) of Sprd-1 and -2 did not differentiate into myotubes even in differentiation medium, and a higher level of MAP kinase activity was maintained (Fig. 3d, e). Thus, ΔC mutants inhibited C2C12 cell differentiation probably by augmenting MAP kinase signalling, supporting the hypothesis that endogenous Sprd proteins function as suppressors of the MAP kinase pathway in various systems.

Next, we investigated the molecular mechanism by which Sprd suppresses the Ras–MAP kinase pathway. As one of the nuclear targets of MAP kinase is Elk-1, a transcription factor of the Ets family, EGF-induced activation of MAP kinase can be monitored by measuring the rate of Elk-1-dependent transcription14. In 293 cells, forced expression of Sprd-1 or -2 dose-dependently suppressed EGF-dependent Elk-1 activation (Supplementary Information Fig. 3a). The negative effect of Sprd-1 and -2 was comparable to that of Ras GTase activating protein (rasGAP) and N17-Ras, and Sprd-1 and -2 were more potent inhibitors than was murine Sprouty-4 or the Raf kinase inhibitor protein (RKIP)15. Both EVH1 and SPR domains were necessary for the suppression of Elk-1 activation. Replacement of the EVH1 domain of Sprd-1 with that of Wiskott–Aldrich syndrome protein (WASP) abolished the inhibitory activity of Sprd-1 (Supplementary Information Fig. 3c), suggesting that the EVH1 domain of Sprd-1 may interact with a specific target required for suppression of the MAP kinase pathway. In contrast, the KBD region was not essential but required for efficient suppression of the MAP kinase pathway (Supplementary Information Figs 2b and 3c).

Figure 3 Effects of wild-type (WT) and C-terminal-truncated (ΔC) Sprd proteins on differentiation of C2C12 cells and Erk2 activation. a, C2C12 cells were cultured in differentiation medium (2% HS) for the indicated periods and Sprd-1 mRNA was measured. b, c, Wild-type Sprd-1, -2 and N17-Ras constructs were transfected with EGFP (b) or Flag-tagged Erk2 (c) then cultured in the growth medium (20% FBS) for 5 d (b) or 2 d (c). Cell morphology (b) and Erk2 phosphorylation (c) were measured. d, e, ΔC Sprd-1 and Sprd-2 constructs and constitutively activated Raf (ΔN-Raf) were transfected with EGFP (d) or Flag-tagged Erk2 (e). Cells were cultured in differentiation medium (2% HS) for 5 d (d) or 2 d (e).
We determined which component of the Ras–MAP kinase pathway is suppressed by Spred. Ras directly interacts with and activates Raf. Raf phosphorylates and activates MEK, which in turn phosphorylates and activates MAP kinases. Spred inhibited activation of Elk-1 induced by active Ras (V12-Ras), but not that induced by active MEK or active Raf (∆N-Raf) (Supplementary Information Fig. 3b). Therefore, the target of Spred is probably located between Ras and Raf. To test this hypothesis, we examined the effect of Spred-1 on EGF-induced Ras and Raf activation (Fig. 4a). Interestingly, Spred sustained Ras activation, whereas it inhibited Raf activation, as measured by autophosphorylation (Fig. 4a) and by in vitro kinase assay (Fig. 4b). Furthermore, like rasGAP, Spred inhibited the phosphorylation of Raf on Ser 338, which is required for Raf activation, but not on Ser 259, which is not (refs 13, 14; Fig. 4c). Thus, Spred inhibits MAP kinase activity by suppressing Raf activation. In contrast, Spred did not affect EGF- or V12-Ras-dependent Akt activation, or EGF-dependent tyrosine phosphorylation of phospholipase-Cγ and membrane ruffling induced by the small GTP-binding protein Rac11 (Supplementary Information Fig. 4). These data suggest that Spred specifically suppresses the Ras–Raf signalling pathway.

We then examined the interaction between Raf, Ras and Spred in C2C12 cells. After immunoprecipitation, endogenous Spred-2 was identified as a protein with a relative molecular mass of 40,000 (Mr 40K) that co-immunoprecipitated with endogenous Ras but not Raf (Fig. 5a). Furthermore, Ras and Spred co-localized at the plasma membrane, independent of EGF stimulation (Fig. 5b). Unexpectedly, Raf was translocated into the plasma membrane even in the presence of Spred-1 and co-localized with Spred-1 (Fig. 5b), thus indicating that inhibition of Raf activation by Spred is not due to a simple masking of the effector domain of Ras. We therefore examined the effect of Spred on the interaction between Raf and activated Ras (V12-Ras). As shown in Fig. 5c, the amount of Raf co-precipitated with active Ras was increased by Spred-1 co-expression. Augmentation of the interaction between Raf and Ras by Spred was confirmed by monitoring the time course of EGF-induced translocation of Raf to the plasma membrane. Raf was retained longer in the plasma membrane by overexpression of Spred-1 or Spred-2 (Supplementary Information Fig. 5). Thus Spred proteins potentiate Ras–Raf interaction, and Raf may not be accessible to Raf kinase.

Figure 4 Spred inhibits the activation of MAP kinase by suppressing Raf activation.

a. Time course of Erk2, Ras and Raf activation in 293 cells transfected with HA-Erk2, wild-type Flag-Ras and Flag-Raf with or without Spred-1. Activated Ras was precipitated with GST–Ras binding domain (GST-RBD) beads, and Raf activation was assessed by an in vitro autophosphorylation (AutoP) assay. b. Suppression of Raf kinase activity confirmed by an in vitro kinase assay using MAP kinase cascade components and MBP. c. Effect of Spred-1 on Raf phosphorylation. Flag-Raf was immunoprecipitated (IP) and blotted (IB) with anti-phospho-Ser 338 (PS338) or Ser 259 (PS259) Raf antibodies.

Figure 5 Interaction of Spred with Ras and Raf. a. Anti-Spred-2 immunoprecipitates (IP) from C2C12 cells were blotted (IB) with the indicated antibodies. TCL, total cell lysates. b. C2C12 cells were transfected with the EGF receptor, Myc-Spred-1 and Flag-Ras, or Myc-Spred-1, Flag-Raf and wild-type (WT) Ras. After stimulation with EGF for 5 min, Myc-tagged and Flag-tagged proteins were stained with secondary antibodies conjugated with FITC (green) and Cy3 (red), respectively. Arrows indicate co-localization. c. C2C12 cells were transfected with Flag-tagged wild-type (WT) or constitutively activated (V12) Ras, Myc-tagged Spred and Myc-tagged Raf. Anti-Flag immunoprecipitates were blotted with anti-Myc and anti-Flag antibodies.
when associated with the Spred–Ras complex, thereby preventing its activation.

*Drosophila* Sprouty is induced by FGF and inhibits Ras–Raf activation by interacting with rasGAP and Drk/Grb2 (ref. 3). The Spred orthologue of *Drosophila* is AE33, which was cloned as a probable target of the rough transcription factor that regulates photoreceptor cell development16. Thus, Spred and Sprouty are evolutionarily conserved, and several isoforms have been identified in mammals. Spred and Sprouty may negatively regulate the Ras–MAP kinase pathway in response to different stimuli in different tissues or organs.

**Methods**

**Screening and cloning of Spred-1 and -2**

Randomly primed cDNA synthesized from mouse osteoclast messenger RNA was cloned into a phage pASV3 vector and packaged. Plasmids were recovered by infecting phage into *Escherichia coli* BNN-132 (ref. 17). The yeast two-hybrid screen was done as described18.

**Plasmid construction**

Mouse Spred-1 and -2, human Ras, Raf and MEK, and murine Sprouty-4 cDNAs were cloned into pCDNA with a six-repeated Myc tag or CMV2 with a Flag tag at the N terminus. Spred-1 and -2 were also cloned into pEGFP (Clontech) to introduce the EGFP tag at the N terminus. Erk2 MAP kinase tagged with haemagglutinin (HA) and Flag was a gift of Y. Goto. WASP cDNA was donated by H. Miike and T. Takenawa. Deletion, substitution and chimeric mutants were generated by standard polymerase chain reaction, as described19.

**Differentiation of PC12 and C2C12 cells**

Rat pheochromocytoma-derived PC12 cells were maintained in DMEM supplemented with 10% FBS and 5% HS. We transfected PC12 cells seeded in plates coated with poly-L-lysine with EGFP-tagged Spred-1, IGF receptor and HA-tagged Erk2, using TransFast (Promega). Myc-tagged Spred-1 was introduced into PC12 cells using a retrovirus vector (pMIX-IRIS-EGFP) carrying Spred-1 cDNA and IRES-EGFP16. From 48 h after transfection or infection, PC12 cells were cultured in the presence of NGF or EGF for 12 h, then examined by fluorescence microscopy. Cells with processes longer than 1.5 times the diameter of the cell body were considered to be positive for neurite outgrowths20. Affinity-purified Spred antibodies with or without antigen peptides were microinjected with Eppendorf transistor 5246 or 5171 and examined by inverted microscopy (Zeiss), as described16. *In situ* Erk phosphorylation assay was performed as described21.

**Immunochrome analysis**

Immunoprecipitation and immunoblotting were performed with anti-Myc (9E10), anti-Flag (ME), anti-Ras (Calbiochem), anti-Erk2 (Santa Cruz), anti-HA (12CA5), anti-phosphorylated-Erk2 (Promega), anti-phospho-Ser 338 (Upstate) or Ser 259 Raf (New England antibodies), as described22. The Ras-GTP form was precipitated with the Raf N-terminal Ras-binding domain fused to GST, as described23. Anti-Spred-1 or -2 antibody was prepared by immunizing rabbits with the KLH-conjugated peptides FKRRKREDKG ERSRC or IKTQPPRASKRRRKENEGC, respectively.

**Kinase assays**

*In vitro* kinase assay for Erk2 and Raf was performed using myelin basic protein as a substrate according to methods described21.

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**Supplementary information** is available on Nature’s World-Wide Web site (http://www.nature.com/) or as paper copy from the London editorial office of Nature.

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**General transcription factors bind promoters repressed by Polycomb group proteins**

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To maintain cell identity during development and differentiation, mechanisms of cellular memory have evolved that preserve transcription patterns in an epigenetic manner. The proteins of the Polycomb group (PCG) are part of such a mechanism, maintaining gene silencing. They act as repressive multiprotein complexes that may render target genes inaccessible to the transcriptional machinery1, inhibit chromatin remodelling2, influence chromosome domain toplogy3 and recruit histone deacetylases (HDACs)4. PCG proteins have also been found to