Gene Disruption of Spred-2 Causes Dwarfism*

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The impact of the fibroblast growth factor receptor 3 (FGFR3)-mediated signaling pathway on bone growth has been demonstrated by various genetic approaches. Overexpression of fibroblast growth factors (FGFs), several gain-of-function mutations in the FGFR3, and constitutive activation of mitogen-activated protein kinase (MAPK) kinase (MEK1) in chondrocytes have been shown to cause dwarfism in mice by activation of the MAPK signaling pathway. To investigate the previously reported inhibitory role of Spred in the FGFR3/MAPK pathway, we generated mice with a trapped Spred-2 gene. Here we show that lack of functional Spred-2 protein in mice caused a dwarf phenotype, similar to achondroplasia, the most common form of human dwarfism. Spred-2−/− mice showed reduced growth and body weight, they had a shorter tibia length, and showed narrower growth plates as compared with wild-type mice. We detected promoter activity and protein expression of Spred-2 in chondrocytes, suggesting an important function of Spred-2 in chondrocytes and bone development. Stimulation of chondrocytes with different FGF concentrations showed earlier and augmented ERK phosphorylation in Spred-2−/− chondrocytes in comparison to Spred-2+/+ chondrocytes. Our observations suggest a model in which loss of Spred-2 inhibits bone growth by inhibiting chondrocyte differentiation through up-regulation of the MAPK signaling pathway.

Long bone growth is determined mainly by the process of endochondral ossification, a strictly regulated process that requires proliferation and differentiation of chondrocytes. During this process, chondrocytes in the reserve zone that arise from mesenchymal cells first undergo proliferation, they then exit the cell cycle, undergo terminal hypertrophic differentiation, and finally the synthesized cartilage matrix calcifies and is replaced by bone (1). Various signaling molecules have been shown to regulate and coordinate this complex process of endochondral ossification. Fibroblast growth factor (FGF)1 signals playing a major role in a variety of developmental processes and recent results have highlighted its function in the regulation of bone morphogenesis (for review, see Ref. 2). FGFs are a large family of at least 23 polypeptides that signal through their binding to specific tyrosine kinase receptors (FGFRs), which constitute a four-member gene family (3). FGF receptor 3 (FGFR3) is expressed in proliferating and prehypertrophic chondrocytes in the epiphyseal growth plates (4–6). Activating mutations in FGFR3 cause different forms of human dwarfism like achondroplasia, hypochondroplasia, and thanatophoric dysplasia (7–10). The most common form of human dwarfism is achondroplasia with a prevalence at birth of about 1/26,000 (11). Expression of activating FGFR3 mutants in mice reproduces the dwarf phenotype of these skeletal diseases (5, 12–17). In contrast, lack of FGFR3 in mice causes skeletal overgrowth, indicating that FGFR3 signaling inhibits endochondral bone growth (18, 19). Similarly, transgenic mice overexpressing FGFs show dwarfism (20, 21), whereas mice homozygous for a targeted disruption of FGFR3 exhibit a growth plate phenotype similar to that of FGFR3 null mice (6, 22). These observations indicate that FGF and FGFR3 signaling play major roles in the regulation of bone growth. To date, four signaling pathways have been shown to propagate FGF3 signals: the STAT, MAPK-extracellular signal-regulated kinase (ERK), phospholipase C-γ, and phosphatidylinositol 3-kinase-AKT pathways (22–31).

Recently, it has been shown that constitutive activation of MEK1 in chondrocytes causes Stat1-independent achondroplasia-like dwarfism in mice and rescues the FGFR3-deficient mouse phenotype (32). Overexpression of C-type natriuretic peptide in chondrocytes counteracts dwarfism in a mouse model of achondroplasia with activated FGFR3 in cartilage. C-type natriuretic peptide prevented the shortening of achondroplastic bones by correcting the decreased extracellular matrix synthesis in the growth plate through inhibition of the FGF-mediated MAPK pathway (33). Growth hormone therapy in achondroplasia patients has beneficial effects through insulin-like growth factor-1 by preventing chondrogenic cell apoptosis induced by an activating mutation in the FGFR3 (34). These results indicate that MAPK activity is a negative regulator of bone growth and suggest that the MAPK pathway plays an important role in bone development. Despite recent advances in understanding the roles of FGFs and FGFR receptors in fibroblast growth factor receptor; ERK, extracellular signal-regulated kinase; STAT, signal transducers and activators of transcription; X-gal, 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside; MAPK, mitogen-activated protein kinase; EVH1, Eno/vasodilator-stimulated phosphoprotein homology-1 domain; KBD, c-Kit-binding domain; SPR domain, Sprouty-related domain; WT, wild-type; RT, reverse transcriptase; VASP, vasodilator-stimulated phosphoprotein; ES cell, embryonic stem cell.

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† The abbreviations used are: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; ERK, extracellular signal-regulated kinase; STAT, signal transducers and activators of transcription; X-gal, 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside; MAPK, mitogen-activated protein kinase; EVH1, Eno/vasodilator-stimulated phosphoprotein homology-1 domain; KBD, c-Kit-binding domain; SPR domain, Sprouty-related domain; WT, wild-type; RT, reverse transcriptase; VASP, vasodilator-stimulated phosphoprotein; ES cell, embryonic stem cell.

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Dwarfism in Spred-2/− Mice

To test for RNA integrity, a glyceraldehyde-3-phosphate dehydrogenase fragment was amplified in parallel, using the following primers: glyceraldehyde-3-phosphate dehydrogenase forward, 5'-ACCAGCTCAGTGCCATCAC-3', and glyceraldehyde-3-phosphate dehydrogenase reverse, 5'-TACGCCGGGTCTTGCCGTTTACC-3'.

For northern blot analysis, 20 μg of each total RNA sample was sequenced nonspecific binding. Sections were incubated with primary antibodies overnight at room temperature, washed 3 times in phosphate-buffered saline, 0.1% Tween 20. After dehybridization, the decalcified tissue were embedded in methylmethacrylate, and 5-μm sections were cut in the sagittal plane on a rotation microtome (Cut 4060E, MicroTech, Munich, Germany). Sections were stained with toluidine blue and evaluated using a Carl Zeiss microscope (Carl Zeiss, Jena, Germany) as described (47).

Estimation of Bone Lengths—To measure bone lengths, isolated legs were examined by soft x-rays. Statistical analyses were calculated by Student's t-test.

Cell Culture—Sterni and costal cartilage were dissected from pups at P1. Tissues were cut into small pieces and incubated in 1 mg/ml collagenase A (Roche) in Dulbecco's modified Eagle's medium for 3 h at 37 °C. After dehydrolysis, the decalcified tissue were embedded in methylmethacrylate, and 5-μm sections were cut in the sagittal plane on a rotation microtome (Cut 4060E, MicroTech, Munich, Germany). Sections were stained with toluidine blue and evaluated using a Carl Zeiss microscope (Carl Zeiss, Jena, Germany) as described (47).

Genotyping of Mice—Offspring were genotyped using the following sets of primers: for WT PCR, amplifying a 1600-bp fragment, primer 1 (5'-GCTTCCGCGCCACCCCGGTAG-3') and primer 2 (5'-TAGAACAAACGTCGATACTGTG-3'), for knockout PCR, amplifying a 2700-bp fragment, primers 1 and 2 (5'-TTCTTGTTTTCGGACACCTG-3').

Spreds have been described as a new Sprouty-related family of membrane-associated physiological inhibitors of the Ras/MAP kinase pathway by interacting with Ras and Raf-1 (35). Spreds contain an N-terminal EVH-1 (Ena/vasodilator-stimulated phosphoprotein homology-1) domain, a C-terminal Sprouty-like cysteine-rich domain (SPR domain). Sprouty from Drosophila was identified as a negative regulator of growth factor-induced ERK activation (36–38), and Spred-1 and Spred-2 are also known inhibitors of FGF and epidermal growth factor signaling (39–41). So far, three mammalian Spred family members have been identified: Spred-1 was found to be expressed in fetal tissues, brain, heart, and to a minor extent in other tissues. Spred-2 expression was found to be more ubiquitous, whereas Spred-3 expression was restricted to the brain (41, 42). During rat lung development, Spred-1 and Spred-2 mRNAs were shown to be expressed predominantly in mesenchymal tissue from the onset of lung branching and co-expression of FGF-10 was demonstrated in the same region (43). In the aorta- gonado-mesonephros region of middigestion mouse embryos, Spred-2 activity negatively regulates aorta-gonado-mesonephros hematopoiesis (44).

Additionally, it was shown that Spreds play a role as key regulators of RhoA-mediated cell motility and signal transduction. Spred-1 binds to activated RhoA and inhibits chemokine-induced RhoA activation and active RhoA-induced Rho kinase activation. By this mechanism, Spred inhibits cell motility, metastasis, and Rho-mediated actin reorganization (40). Recently, it has been demonstrated that Spred-1 is also highly expressed in hematopoietic cells and negatively regulates stem cell factor- and interleukin 3-mediated ERK/MAP kinase activation. Wild-type Spred-1 inhibits colony formation of bone marrow cells in the presence of cytokines, whereas a ΔC- Spred-1 dominant negative mutant enhances colony formation (45). Moreover, Spred-1 is expressed in eosinophils and negatively regulates allergen-induced airway eosinophilia and hyper-reactiveness (46).

So far, a function of Spred in the regulation of bone growth has not been shown and the in vivo functions of Spred in the whole organism still remained to be elucidated. Therefore, we generated a mouse strain with a disruption of the Spred-2 gene. In this study, we demonstrate the importance of Spred-2 as an inhibitor of the FGF-induced MAPK signaling pathway in regulation of endochondral ossification during bone development.

EXPERIMENTAL PROCEDURES

Gene Trapping and Southern Blot Analysis—To disrupt the Spred-2 gene in mice, we generated a mouse strain, using the XR228 embryonic stem cell line with the pGTO gene trap vector inserted between exons 4 and 5 (Baygenomics, San Francisco, CA). Vector insertion was confirmed by X-gal staining according to the protocol given by the Sanger Institute Gene Trap Resource (www.sanger.ac.uk/PostGenomics/gentrap/protocols.shtml), and single insertion of the gene trap vector was confirmed by Southern blot analysis. The Southern probe was a 630-bp fragment of the engrailed 2 intron 1 gene trap vector sequence, which was amplified with the following primers: forward, 5'-AGATGCAGAGACCTCACTTGAAGCC-3', and reverse, 5'-TCTTTGTTGTTTCCGAGACCTGG-3'. 10 μg of genomic XB228 DNA was digested with BglII, and 5 (Baygenomics, San Francisco, CA). Vector insertion was confirmed by X-gal staining according to the protocol given by the Sanger Institute Gene Trap Resource (www.sanger.ac.uk/PostGenomics/gentrap/protocols.shtml), and single insertion of the gene trap vector was confirmed by Southern blot analysis. The Southern probe was a 630-bp fragment of the engrailed 2 intron 1 gene trap vector sequence, which was amplified with the following primers: forward, 5'-AGATGCAGAGACCTCACTTGAAGCC-3', and reverse, 5'-TCTTTGTTGTTTCCGAGACCTGG-3'.

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For histopathology, skeletons were fixed in 3.7% formaldehyde for 6 weeks. For X-gal stainings, newborn mice and tibiae of P7 were prepared in Laemmli buffer. Proteins were separated by SDS-PAGE on 10 or 12% gels and transferred to nitrocellulose membranes using a semi-dry system (Bio-Rad). For Western blot analyses, the following antibodies were used: affinity purified polyclonal rabbit anti-Spred-2 (AS 96–415 (42)), polyclonal goat anti-actin (catalog number sc-1616, Santa Cruz Biotechnology), polyclonal rabbit anti-ERK (catalog number EP127, Cell Signaling Technology), monoclonal rabbit anti-phospho-ERK (catalog number 4376, Cell Signaling Technology), peroxidase-conjugated goat anti-rabbit IgG (Dianova), and peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology). For signal detection, the ECL kit (Amersham Biosciences) was used.
RESULTS

Generation of Spred-2-deficient Mice—To generate Spred-2-deficient mice, an embryonic stem cell clone with a disrupted Spred-2 gene, XB228 (Baygenomics), was used for blastocyst injection. Chimeric male offspring were then mated to wild-type C57Bl/6 mice to test for germ line transmission of the Spred-2 gene. The gene trap vector pGTO in the XB228 ES cell line was used as the radioactive probe (Fig. 1A). Appearance of only one band in the blot, which was also present in wild-type DNA, is indicative of single insertion of the gene trap vector. The upper band in the blot, which was also present in wild-type DNA, corresponds to the endogenous engrailed 2 (en2) gene. C, representative PCRs giving genotype of mice: knock-out and wild-type. M, size marker.

by PCR analyses using primers 1 and 2 (Fig. 1A) for the knock-out PCR and primers 1 and 3 (Fig. 1A) for the wild-type PCR (Fig. 1C).

Male and female knock-out mice were fertile and inbred matings resulted in viable offspring, although litters were smaller (average litter size 4.4 pups, ranging from 3 to 7), compared with litters of wild-type breeding pairs (average 8.4, ranging from 5 to 12). In general, life expectancy, nesting, and nursing behavior of knock-out mice was indistinguishable compared with their littersmates.

Spred-2 Deficiency in Knock-out Mice on RNA and Protein Level—Successful disruption of the Spred-2 gene in Spred-2−/− mice was tested at both RNA and protein levels. Total RNA of different organs derived from Spred-2-deficient and wild-type mice was subjected to RT-PCR using specific primers for Spred-2 and glyceraldehyde-3-phosphate dehydrogenase. Loss of Spred-2 mRNA was confirmed in all samples tested (Fig. 2A). Northern blot analyses of brain, representing the organ with the strongest natural Spred-2 expression, revealed no apparent forms of Spred-2 mRNAs in total RNA preparations of Spred-2−/− mice (Fig. 2B). To examine Spred-2 deficiency at the protein level, Western blots were performed with affinity purified polyclonal rabbit anti-Spred-2 antibodies. As above, brain lysates of wild-type and Spred-2−/− mice were used as indicator
Dwarfism in Spred-2\(^{-/-}\) Mice

**Dwarfism in Spred-2\(^{-/-}\) Mice**—To determine whether the dwarf phenotype might be because of changed levels of steroid or thyroid hormones, we compared serum levels of testosterone, estrogen, thyroid stimulating hormone, 3,5,3′-triiodothyronine, and thyroxin in female and male knock-out and wild-type mice. None of these investigated hormone levels was found to be altered and, therefore, altered hormone production and secretion of steroid and thyroid hormones could be excluded (data not shown). As dwarfism could also be because of changed insulin metabolism, we tested the blood glucose levels of Spred-2\(^{-/-}\) and Spred-2\(^{-/-}\) mice at different times of the day. Because no significant alterations in glucose levels were detectable, a dysfunction of insulin metabolism seemed very unlikely (data not shown).

To observe whether the lack of a functional Spred-2 protein causes structural changes in organs of these mice, we stained paraffin sections of several organs with hematoxylin and eosin. Apart from a clear increase in number of megakaryocytes in the spleen of Spred-2 knock-out mice as compared with wild-type littermates, we did not observe obvious structural changes in lung, heart, liver, kidney, stomach, small intestine, large intestine, thymus, testis, ovaries, uterus, skeletal muscle, salivary glands and the prostate (data not shown).

**Spred-2 Promoter Activity in Bones**—Assuming that the dwarf phenotype was because of a skeletal disorder, we determined whether Spred-2 was expressed in bones and chondrocytes. To visualize the expression pattern of Spred-2, we analyzed the activity of the artificial β-gal gene fusion vector of the pGTO gene trap vector, which is, following appropriate integration of the vector, brought under the control of the Spred-2 gene promoter. X-gal stainings of the lower legs of P7 knock-out and wild-type mice (Fig. 4, A–H) revealed Spred-2 promoter activity in chondrocytes. Strong activity was found in the whole tibia growth plate (Fig. 4A) and the peristeam (Fig. 4, A and G). Spred-2 expression was seen in chondrocytes of all corresponding cartilage areas of the knee joint including the patella, tibia, and femur (Fig. 4C).

Remarkable Spred-2 expression was also seen in secondary ossification centers of long bones (Fig. 4E) and in distinct cells of bone marrow (Fig. 4G). As negative controls, corresponding wild-type sections were examined (Fig. 4, B, D, F, and H). Additional cryosections of newborn knock-out mice were stained with X-gal, and Spred-2 promoter activity was detected in ribs (Fig. 4J), in chondrocytes of the acetalbulum, the femur head in the hip joint (Fig. 4K), and in secondary ossification centers of the femur (Fig. 4L). In a higher magnification, X-gal activity was clearly visible in chondrocytes of the tibia growth plate (Fig. 4L). X-Gal staining of thoraxes of adult mice revealed Spred-2 expression at the cartilage areas of the ribs (Fig. 4M).

**Expression of Endogenous Spred-2 in Chondrocytes**—To investigate endogenous Spred-2 protein expression, lower legs of P7 wild-type mice were dissected and immunostained with a polyclonal Spred-2-specific antibody. Thereby, we confirmed that Spred-2 protein expression was congruent to the detected Spred-2 promoter activity shown in Fig. 4. Spred-2 was found to be expressed in chondrocytes of growth plates (Fig. 5E), the tibia head (Fig. 5F), secondary ossification centers (Fig. 5G), the periostium, and distinct cells in the bone marrow (Fig. 5H). Corresponding sections were stained with secondary antibody as negative controls (Fig. 5, A–D).

**Reduced Tibia Length and Narrower Growth Plate in Spred-2-deficient Mice**—Soft x-ray exposures of wild-type and knock-out lower legs showed a highly significant reduction in tibia length of Spred-2-deficient mice (Fig. 5, I and J, average WT = 18.1 mm, ranging from 17 to 21 mm; average knock-out = 16.1

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**Fig. 2. Loss of Spred-2 in XB228-derived mice.** A, RT-PCR showing loss of full-length Spred-2 mRNA in selected organs of knock-out (ko) mice in comparison to wild-type controls (wt). B, Northern blot using wild-type and knock-out brain RNA. Note that no truncated or prolonged band was detectable in knock-out mouse-derived RNA preparations. C, Western blot demonstrating lack of Spred-2 protein in brain lysates of Spred-2\(^{-/-}\) mice in comparison to wild-type control mice. Actin levels indicate equal loading, a lysate of Spred-2-overexpressing HEK293 cells served as positive control, and a lysate of Spred-1-overexpressing HEK293 cells demonstrate the Spred-2 antibody specificity. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Table 1.** Loss of Spred-2 in XB228-derived mice.

| Organ          | Wild-type (wt) | Knock-out (ko) | WT/ko ratio |
|----------------|----------------|----------------|-------------|
| Lung           | 0.6% ± 0.137%  | 0.8% ± 0.081%  | 1.30        |
| Heart          | 0.4% ± 0.119%  | 0.5% ± 0.076%  | 1.14        |
| Kidney         |                |                |             |
| Liver          | 0.4% ± 0.095%  | 0.6% ± 0.099%  | 1.14        |
| Spleen         | 5.0% ± 0.707%  | 3.8% ± 0.391%  | 1.30        |
| Average        | 0.6% ± 0.137%  | 0.8% ± 0.081%  | 1.30        |

**Fig. 3.**

A: A time course depicting body weight differences between male wild-type and knock-out mice. Actin levels indicate equal loading, a lysate of Spred-2-overexpressing HEK293 cells served as positive control, and a lysate of Spred-1-overexpressing HEK293 cells demonstrate the Spred-2 antibody specificity. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

B: Western blot demonstrating lack of Spred-2 protein in brain lysates of Spred-2\(^{-/-}\) mice in comparison to wild-type control mice. Actin levels indicate equal loading, a lysate of Spred-2-overexpressing HEK293 cells served as positive control, and a lysate of Spred-1-overexpressing HEK293 cells demonstrate the Spred-2 antibody specificity. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Fig. 4.** X-Gal stainings of the lower legs of P7 knock-out and wild-type mice (Fig. 4, A–H) revealed Spred-2 promoter activity in chondrocytes. Strong activity was found in the whole tibia growth plate (Fig. 4A) and the peristem (Fig. 4, A and G).

**Fig. 5.** X-Gal stainings of the lower legs of P7 knock-out and wild-type mice (Fig. 5E) and in distinct cells in the bone marrow (Fig. 5H).
mm, ranging from 14 to 18 mm; $n_{WT} = 32$; $n_{knock-out} = 26$; $p < 0.01$). To investigate the morphology of growth plates, we examined the histology of epiphyseal growth plates at 1, 4, and 12 weeks of knock-out and control mice. Growth plates of knockout mice showed narrower zones of hypertrophic chondrocytes, and the proliferative and hypertrophic zone together was shorter in all stages compared with wild-type mice (Fig. 6).

Increased and Earlier ERK Phosphorylation in Spred-2 KO Chondrocytes after FGF Stimulation—Because Spred-2 has been shown to be a negative regulator of the MAPK signaling pathway (35), we examined ERK and phosphorylated ERK in chondrocytes. Stimulation of cultured chondrocytes with different concentrations of FGF (5, 10, and 50 ng/ml) revealed an earlier and increased ERK phosphorylation in Spred-2 KO chondrocytes in comparison to Spred-2 WT chondrocytes. Non-specific stimulation with fetal bovine serum (1 or 10% FCS) did not result in altered ERK phosphorylation in chondrocytes of wild-type and knockout chondrocytes (Fig. 7).

**DISCUSSION**

**Domain Structure of Spred-2**—Besides the Drosophila AE33 gene, cloned as a probable rough transcription factor target regulating photoreceptor cell development in the fly (48), three mammalian Spred proteins have recently been described as negative regulators of growth factor-induced MAPK pathways (35, 41). They consist of three distinct domains, namely the N-terminal EVH-1 (Ena/vasodilator-stimulated phosphoprotein (VASP) homology) 1 domain, a unique KBD domain, and a C-terminal SPR (Sprouty-related) domain. EVH-1 domains are protein interaction modules that target their multidomain host proteins to specific sites of action where they are involved in regulating cellular processes as diverse as cytoskeletal reorganization, synaptic transmission, proliferation, and differentiation (reviewed in Refs. 49 and 50). The KBD is not related to any previously identified tyrosine kinase interaction domain, such as SH2, phosphotyrosine-binding domain, or c-Met-binding domain, and this domain, consisting of about 50 amino acids, was required for efficient phosphorylation of Spred-1 (35). The cysteine-rich SPR domain involved in membrane localization was first described in the context of Sprouty proteins, which have been shown to be both positive and negative regulators of MAPK pathways (for review, see Refs. 51 and 52), and the efficient suppression of ERK activation by Spred requires this SPR domain (41). Here we show, at the DNA level, the schematic organization of the six exons encoding Spred-2 and the correlated domain structure at the protein level. The mouse Spred-2 is located on chromosome 11 (41); exon 1 to exon 3 code for the EVH-1 domain, exon 4 and exon 5 code for the middle part, and exon 6 is responsible for the KBD and SPR domain (Fig. 1A).

**Physiological Function of Spred**—Sprouts selectively inhibit FGF-induced ERK activation but do not inhibit epidermal growth factor-induced ERK activation (39). In contrast, overexpression of Spred-1 and Spred-2 efficiently suppressed ERK activation induced by several stimuli, including epidermal growth factor, FGF, vepidermal growth factor, platelet-derived growth factor, stem cell factor, serum and lysophosphatidic acid. Spred constitutively associates with Ras and inhibits the activation of MAP kinase by suppressing phosphorylation and activation of Raf (35). First insights in the in vivo functions of Spred proteins came from studies on isolated hematopoietic cells derived from midgestation Spred-2–/– mice, showing an increased number of granulocyte and macrophage colonies (44). In bone marrow-derived mast cells of Spred-1–/– mice an augmentation of ERK activation and proliferation in response to interleukin-3 was observed (45). Recently, it has been shown that Spred-1 is expressed in eosinophils and negatively regu-
lates allergen-induced airway eosinophilia and hyperresponsiveness (46).

Except for these data, all functional information about Spred was gained by overexpression of full-length constructs or deletion mutants in different cell culture systems. Therefore, the observed general inhibitory effects of Spred proteins on the Ras/MAP kinase pathways might be because of high levels of overexpressed proteins and may not necessarily reflect the in vivo situation in which functional interaction is strongly dependent on specific expression levels and affinity of interacting proteins. So far, no comprehensive in vivo data of Spred function were available. Therefore, our Spred-2/H11002 mouse line represents a new in vivo model to investigate Spred-2 function in the entire organism.

**Gene Trap Models**—In this study we used the ES cell line XB228 harboring a gene trap vector insertion between exons 4 and 5 of the Spred-2 gene. Previously, it has been described in different knock-out strategies that exons in front of the vector insertion were still used as an RNA template and a truncated protein was expressed. Expression of read-through products containing exon and vector information or truncated splice variants has also been reported. As Spred-2 was interrupted downstream of exon 4, expression of the first four exons, basically representing the EVH-1 domain, could not be excluded. Based on sequence comparisons, four different subclasses of EVH-1/WH-1 domains have been identified. High resolution structures of three classes, comprising the cytoskeletal ENA/VASP proteins, the synaptic terminal Homer/Vesl proteins, and the Wiskott-Aldrich syndrome proteins have been solved (for reviews, see Refs. 49 and 53). Recently, the EVH-1 structure of Spred proteins has also been enlightened (54). The EVH-1 domain of VASP was shown to act as a dominant negative form when overexpressed in cardiac myocytes (55). Overexpression of a C-mutant of Spred-1, missing the Sprouty domain but still containing the EVH-1 and the c-Kit binding domains, demonstrated the dominant negative behavior of Spred-EVH1, as well (45). Immunoblot analyses of Spred-2/H11002 mice brains, the organ with the highest natural Spred-2 expression, failed to detect the full-length Spred-2, a truncated Spred-2 protein, or an enlarged read-through product (Fig. 2C). These results were confirmed by Northern blots in which Spred-2 mRNAs in tissues of wild-type mice but no full-length or truncated transcripts in samples of Spred-2/H11002 mice were detectable (Fig. 2B). Based on these findings, we conclude that we have generated Spred-2 null mice that do not express truncated, potentially dominant negative Spred-2 forms.

**Spred-2 Knock-out Mice**—Spred-1 overexpression in osteosarcoma cells inhibited tumor proliferation, metastasis, cell migration, and Rho-dependent actin-stress fiber remodeling (40). Therefore, one could speculate about a phenotype with

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**Fig. 4. Spred-2 promoter activity in bones.** X-gal staining of cryosections of knock-out (A, C, E, and G) and wild-type (B, D, F, and H) lower legs at P7. Corresponding wild-type sections serve as negative controls for specific X-gal staining in knock-out mice. A, X-gal staining shows Spred-2 promoter activity in chondrocytes of the growth plate and strong activity in the periosteum of proximal tibiae (arrows). C, patella and the corresponding area of the tibia show Spred-2 activity in chondrocytes (arrows). Arrows in E indicate expression of Spred-2 in secondary ossification centers of the tibia head. G, positive Spred-2 signal in the perioste of the femur metaphyse is marked by arrows; the arrowheads indicate a strong signal in distinct cells in the bone marrow. I-L, X-gal staining of cryosections of newborn knock-out mice: Spred-2 promoter activity was observed in a secondary ossification center of the distal femur (arrow in I). Longitudinal section through a rib (J) and a femur head in the acetabulum (K) show a strong signal in chondrocytes. Higher magnification demonstrates distinct Spred-2 promoter activity in chondrocytes of the tibia growth plate (L). Scale bars: 200 μm (A-K) and 50 μm (L). M, Spred-2 promoter activity in cartilage areas of ribs of adult Spred-2−/− mice (arrows); and wild-type thorax (left) as negative control.
hyperproliferative cell populations in different organs and tumor development with a forced metastatic situation in Spred-2-deficient mice. In a monitored time period of up to 12 months none of these phenomena appeared in Spred-2-deficient mice. Histology indicated unaltered tissue structures without any tumor formations. Males and females were fertile, were born healthy, and do not display any strange behavior.

We observed an increased number of megakaryocytes in the spleen of adult Spred-2-deficient mice. This has already been shown in Spred-2−/− and Spred-1−/− mice generated by a dif-

FIG. 5. Spred-2 deficiency causes bone growth defects. A–H, immunohistochemistry of wild-type tibiae at P7. A–D, corresponding negative controls to the figures in the second row stained with the secondary antibody alone. E–H, Spred-2 expression is demonstrated in different tibia areas. E shows a Spred-2 signal in chondrocytes of the growth plate, primary spongiosa, and periosteum. In F, the tibia part of the knee joint is shown with Spred-2 expressing chondrocytes in the tibia head. Spred-2 is also present in secondary ossification zones of the tibia head (G), in the periosteum, and the bone formation zone of the tibia metaphyse (H). Distinct cells in the bone marrow have a positive Spred-2 signal (H). I, soft x-rays of wild-type and knock-out lower legs, showing tibia length differences. J, statistical significant differences of tibia lengths of wild-type and knock-out tibiae (*, p < 0.01; n(knock-out) = 26, n(WT) = 32).

FIG. 6. Narrower growth plates in Spred-2-deficient mice. Toluidine staining of tibia growth plates derived from 1-, 4-, and 12-week wild-type (WT) and knock-out (ko) littermates. Measurement of growth plate thickness is marked below each figure. All stages of knock-out mice demonstrate a narrower growth plate, especially with a reduction of the hypertrophic chondrocyte zone, compared with the wild-type controls. wk, week(s); *, p < 0.05; n = 4 animals for each time point.

FIG. 7. Earlier and increased FGF-induced ERK phosphorylation in Spred-2−/− chondrocytes. Chondrocytes of Spred-2−/− and Spred-2−/− mice were stimulated with 5 ng of FGF/ml, 10 ng of FGF/ml, 50 ng of FGF/ml, and 1 and 10% FCS for the indicated periods. Cell extracts were immunoblotted with anti-ERK1/2 or anti-phospho-ERK1/2 antibodies. Phosphorylation of ERK is earlier and increased in stimulated Spred-2−/− chondrocytes with all tested FGF concentrations. (Chondrocyte stimulations were done in four different WT/knock-out pairs of mice.)
Different knock-out strategy (44, 45). In VASP$^{-/-}$ mice, a moderate hyperplasia of megakaryocytes has been observed and VASP-deficient platelets have an increased aggregation response to known stimuli and partial resistance to cAMP and cGMP effects (56, 57). Similar to Spred proteins, VASP contains an EVH1 domain at the N terminus, suggesting that this type of domain might also be important in megakaryocyte function.

**Spred-2 Loss of Function Causes Dwarfism—**Our Spred-2$^{-/-}$ mice exhibited an obvious dwarf phenotype. They were born smaller and lighter and stayed smaller throughout their whole life, as compared with wild-type littermates (Fig. 3, A and C–F). In males, growth differences were more pronounced than in females, which might be an X-chromosomal compensatory effect. Data base analyses revealed a genomic sequence similar to Spred-2 on the mouse X-chromosome (NCBI Gene data base, Loc213280, Chromosome X A4, Contig NT_039702), which might be a putative “Spred-4” or just a pseudogene and has to be investigated further.

Peripheral steroid and thyroid hormones as well as blood glucose levels were not altered. Therefore, we could exclude the most common causes of metabolic dwarfism. X-ray exposures and tibia length measurements revealed that the growth difference was probably because of a defect in skeletal development (Figs. 3B and 5, I and J). Whereas Spred-3 is expressed exclusively in brain, Spred-1 and Spred-2 were found to have an overlapping expression pattern in various tissues (41, 42). Here we show that Spred-2, the most ubiquitously expressed isoform, was expressed in chondrocytes of bones, growing by secondary ossification, suggesting a specific role of Spred-2 in these cells (Figs. 4 and 5, A–H). In growth plates of long bones, where endochondral ossification regulates bone growth, Spred-2 expression was detected in resting, proliferating, and hypertrophic chondrocytes (Figs. 4 and 5, E and G). Tibiae of 1-, 4-, and 12-week Spred-2-deficient mice showed a narrower growth plate and a reduction in size of hypertrophic chondrocytes as compared with wild-type littermates (Fig. 6), indicating a chondrocyte dysfunction at the growth plate during endochondral ossification.

Overexpression of FGFs (20, 21), activating FGFR3 mutants (5, 12–17), or constitutive activation of MEK1 in chondrocytes (32) caused achondroplasia-like dwarfism in mice. This indicates that FGF signaling through the FGFR3 and MAPK pathway plays a major role in the regulation of bone growth. Therefore, it can be regarded as an important negative regulator of skeletal growth. Spred is known to be an inhibitor of FGF-induced MAPK signaling by binding to Ras and inhibiting phosphorylation of Raf-1 (35, 39, 41). In line with this, lack of functional Spred-2 may accelerate MAPK signaling because an inhibitor of the system is removed. In this study, stimulation of cultured Spred-2$^{-/-}$ chondrocytes with different FGF concentrations demonstrated an earlier and increased ERK phosphorylation as compared with wild-type chondrocytes, whereas unspecific stimulation with FCS revealed no differences between wild-type and knock-out cells (Fig. 7). We observed a similar achondroplasia-like dwarfism phenotype in Spred-2$^{-/-}$ mice as described for other factors activating the MAPK pathway. So far, information about downstream events through which FGFs influence the proliferation or differentiation of osteogenic chondrocytes is rare.

In this study, we demonstrate that Spred-2 is an important modulator of bone morphogenesis by inhibiting the FGF-induced MAPK pathway, and loss of Spred-2 causes dwarfism by activating the MAPK pathway in chondrocytes. These observations support the model in which FGFR3 signaling inhibits bone growth by inhibiting chondrocyte differentiation through the MAPK pathway. Recently, a slightly reduced body weight was also mentioned for Spred-1$^{-/-}$ mice, underlining Spred function in growth regulation (46).

Contrary functions in bone growth have been published for Sprouty protein family members, which have also been identified as inhibitors of the MAPK pathway and contain, like Spred, a SPR domain at the C terminus. In contrast to Spred-2, overexpression of vertebrate Sproutys in limbs caused reduction in size of skeletal elements because of an inhibition of chondrocyte differentiation (36). Therefore, Sproutys could act as negative feedback regulators (36), whereas Spred-2 appears to play a role as a downstream inhibitor of FGF-induced MAPK signaling in chondrocytes.

The fairly mild phenotype of Spred-2 knock-out mice is probably because of the compensatory effects of Spred-1, which is co-expressed in various organs (42). Even in brain, which is the organ with the highest expression of Spred-2 in wild-types, Spred-2 deficiency has not caused dramatic disturbances, suggesting compensation by Spred-1 and/or Spred-3.

A comparable situation was described for gene ablation of Mena or VASP proteins. In vertebrates, genetic analysis of Ena/VASP function is hindered by the broad and overlapping expression of the three highly related family members Mena (Mammalian enabled), VASP, and EVL (Ena-VASP like). Mice deficient in either Mena or VASP exhibit subtle defects in forebrain commissure formation and platelet, respectively (56–58). Mena$^{-/-}$/VASP$^{-/-}$ double mutants die perinatally and display defects in neurulation, in development of craniofacial structures, and in the formation of several fiber tracts in the central nervous system and peripheral nervous system (59). It is likely that the expression of the third family member, EVL, masks the requirement for Ena/VASP function in other cell types of Mena$^{-/-}$/VASP$^{-/-}$ animals. In another mouse model, specific overexpression of a C-terminal fragment of VASP in keratinocytes resulted in transgenic mice with skin defects (60). However, cortical lamination and skin defects were not detected in Mena$^{-/-}$/VASP$^{-/-}$ mice, suggesting that continued expression of EVL alone is likely sufficient for proper development of many tissues in these mice. Therefore, it is certainly of interest to generate Spred-1$^{-/-}$ and Spred-2$^{-/-}$ double knock-out mice, or even triple knock-out mice for all three known Spred family members to study the general in vivo function of Spred.

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