Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2

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The brachydactylies are a group of inherited disorders of the hands characterized by shortened digits. Mutations in the tyrosine kinase receptor Ror2 cause brachydactyly type B (BDB). Mutations in GDF5, a member of the BMP/TGF-β ligand family, cause brachydactyly type C (BDC) whereas mutations in the receptor for GDF5, BRI-b, cause brachydactyly type A2 (BDA2). There is considerable degree of phenotypic overlap between the subtypes BDB, BDC and BDA2. Here we demonstrate that all three components are involved in GDF5 induced regulation of chondrogenesis. We show that Ror2 (tyrosine kinase receptor) and BRI-b (serine/threonine kinase receptor) form a ligand independent heteromeric complex. The frizzled-like-CRD domain of Ror2 is required for this complex. Within that complex Ror2 gets transphosphorylated by BRI-b. We show that Ror2 modulates GDF5 signalling by inhibition of Smad1/5 signalling and by activating a Smad-independent pathway. Both pathways however, are needed for chondrogenic differentiation as demonstrated in ATDC5 cells. The functional interaction of Ror2 with GDF5 and BRI-b was genetically confirmed by the presence of epistatic effects in crosses of Ror2, BRI-b and Gdf5 deficient mice. These results indicate for the first time a direct interaction of Ser/Thr- and Tyr-Kinase receptors and provide evidence for modulation of the Smad-pathway and GDF5 triggered chondrogenesis.

Introduction

Ror2 belongs to the receptor tyrosine kinase superfamily, a large family of membrane spanning cell surface receptors that play an important role in the control of many fundamental cellular processes including cell proliferation, cell migration, differentiation, metabolism and survival (Masiakowski & Carroll 1992). Like other receptor tyrosine kinases, Ror2 contains several recognizable structural motifs within the extracellular domain, an immunoglobulin-like domain, a Frizzled-like cysteine-rich domain (CRD), and a membrane-proximal kringle domain. The cytoplasmic region of Ror2 is predicted to contain a conserved tyrosine kinase (TK) domain and, more C-terminal, two regions rich in serine and threonine residues separated by a region rich in proline (for review see Forrester 2002; Yoda *et al*. 2003).

The function of Ror2 has been elucidated by the identification of mutations in human malformation syndromes and by the analysis of mice with inactivated Ror2 alleles. $Ror2^{-/-}$ mice show vertebral malformations and severe shortening of limbs and die perinatal due to respiratory insufficiency (DeChiara *et al*. 2000; Takeuchi *et al*. 2000). Affected mice exhibit many overlapping features with human Robinow syndrome, a recessive condition characterized by mental retardation, shortening of the limbs, vertebral malformations and a characteristic facies. Robinow syndrome is caused by inactivating mutations in *Ror2* (Afzal *et al*. 2000; van Bokhoven *et al*. 2000; Patton & Afzal 2002). Furthermore, brachydactyly type B (BDB), a dominant condition featuring aplasia/hypoplasia of the distal phalanges and/or symphalangism was shown to be caused by specific mutations in *Ror2*

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which result in truncation of the receptor either before or after the TK domain (Oldridge *et al.* 2000; Schwabe *et al.* 2000). The severe skeletal phenotype in $Ror2^{-/-}$ mice as well as the short stature in Robinow patients suggest a role of Ror2 in the differentiation of growth plate chondrocytes. The absent or hypoplastic distal phalanges and the defects in joint formation observed in BDB patients point to a role of Ror2 in early chondrocyte condensation, differentiation and joint formation.

GDF5, a member of the TGF-β/BMP superfamily induces chondroblastic and osteoblastic differentiation and the formation of joints (Storm *et al.* 1994; Francis-West *et al.* 1999). GDF5 is predominantly expressed in the precartilaginous mesenchymal condensations and the cartilaginous cores of the developing long bone. Mutations within the human gene of GDF5 result in a distinct subtype of brachydactyly (BDC) (Polinkovsky *et al.* 1997). Mutations in Gdf5 in the mouse are known as brachypodism (Storm *et al.* 1994). Signalling of the TGF-β superfamily members requires binding of the ligand to a cell surface receptor complex consisting of two types of transmembrane serine/threonine kinase receptors classified as type I and type II (Lutz & Knaus 2002; Shi & Massague 2003). GDF5 binds preferentially to the type I receptor BRI-b whereas BMP-2 binds with high affinity to both BRI-a and BRI-b (Koenig *et al.* 1994; 10 Dijke *et al.* 1994; Nishitoh *et al.* 1996). After ligand binding type II receptor transphosphorylates and thus activates the type I receptor. Intracellular substrates of the activated type I receptors are the Smads. Smads 1, 5 and 8 are phosphorylated by BRI-a or BRI-b and then translocated to the nucleus, where they participate in the transcriptional regulation of genes involved in cartilage and bone formation (Massague 2000; Miyazono *et al.* 2001).

Gene targeting experiments of BRI-b exhibit defects largely restricted to the appendicular skeleton (Yi *et al.* 2000). Mice carrying mutations in *BRI-b* show that this receptor regulates chondrogenesis and segmentation through both GDF5-dependent and -independent processes, and that, reciprocally, GDF5 acts through both BRI-b and other receptors (Baur *et al.* 2000). Most recently we could show that mutations within the gene of BRI-b in human cause Brachydactyly A2 (BDA2) (Lehmann *et al.* 2003).

Here we report that all three components (Ror2, GDF5 and BRI-b), which upon mutations are involved in different types of Brachydactyly, are associated to regulate chondrogenesis. Ror2 modulates signalling of BRI-b by direct interaction of both receptors. This leads to repression of Smad signalling. In addition we show that GDF5 induced chondrogenesis depends on functional Ror2. This is the first evidence for tyrosine kinase receptors directly modulating signalling of BMPs by association with the BMP-receptors.

Results

GDF5 binds with high affinity to BRI-b and low affinity to Ror2

It was shown previously that GDF5 binds most efficiently to BRI-b compared with the other type I receptors (Nishitoh *et al.* 1996). We confirmed binding and chemical crosslinking of iodinated GDF5 in COS7 cells transiently expressing BRI-b-HA. After immunoprecipitation using anti-HA, crosslinked BRI-b was detected (Fig. 1A, lanes 4 and 3). Single expression of Ror2-Flag shows only weak binding or crosslinking of GDF5 after immunoprecipitation with anti-Flag (Fig. 1A, lane 1).

Ror2 and BRI-b associate in the presence and absence of GDF5

Coexpression of both receptors shows that anti-Flag (i.e. anti-Ror2) is able to co-precipitate GDF5 bound BRI-b (Fig. 1A, lane 2), suggesting an association of GDF5 bound to its high affinity receptor BRI-b with Ror2.

The interaction of Ror2 with BRI-b was further investigated by co-immunoprecipitation without crosslinking of iodinated ligand. COS7 cells coexpressing Ror2 and BRI-b were treated with or without GDF5. After cell lysis BRI-b was immunoprecipitated (anti-HA) and associated Ror2 was verified by Western-blotting using anti-Flag antibodies. As shown in Fig. 1B, Ror2 and BRI-b are associated in the absence and presence of GDF5. Other receptors such as BRI-a and BRII show no interaction with Ror2 under the same conditions (Fig. 1C, lanes 2 and 10).

Ligand independent interaction of BRI-b and Ror2 is accompanied by transphosphorylation of Ror2 by BRI-b

In a complementary, however, much more sensitive assay, we measured the *in vitro* kinase activity of co-immunoprecipitated receptors from COS cell lysates. Here we were able to show again that even in the absence of GDF5 both Ror2 and BRI-b are associated in a complex (Fig. 2A, lane 3). To investigate the kinase activity of Ror2 (tyrosine kinase) and BRI-b (serine/threonine kinase) alone or within the heteromeric complex we performed *in vitro* (Fig. 2) and *in vivo* phosphorylation studies (data not shown). Expression of the receptor in

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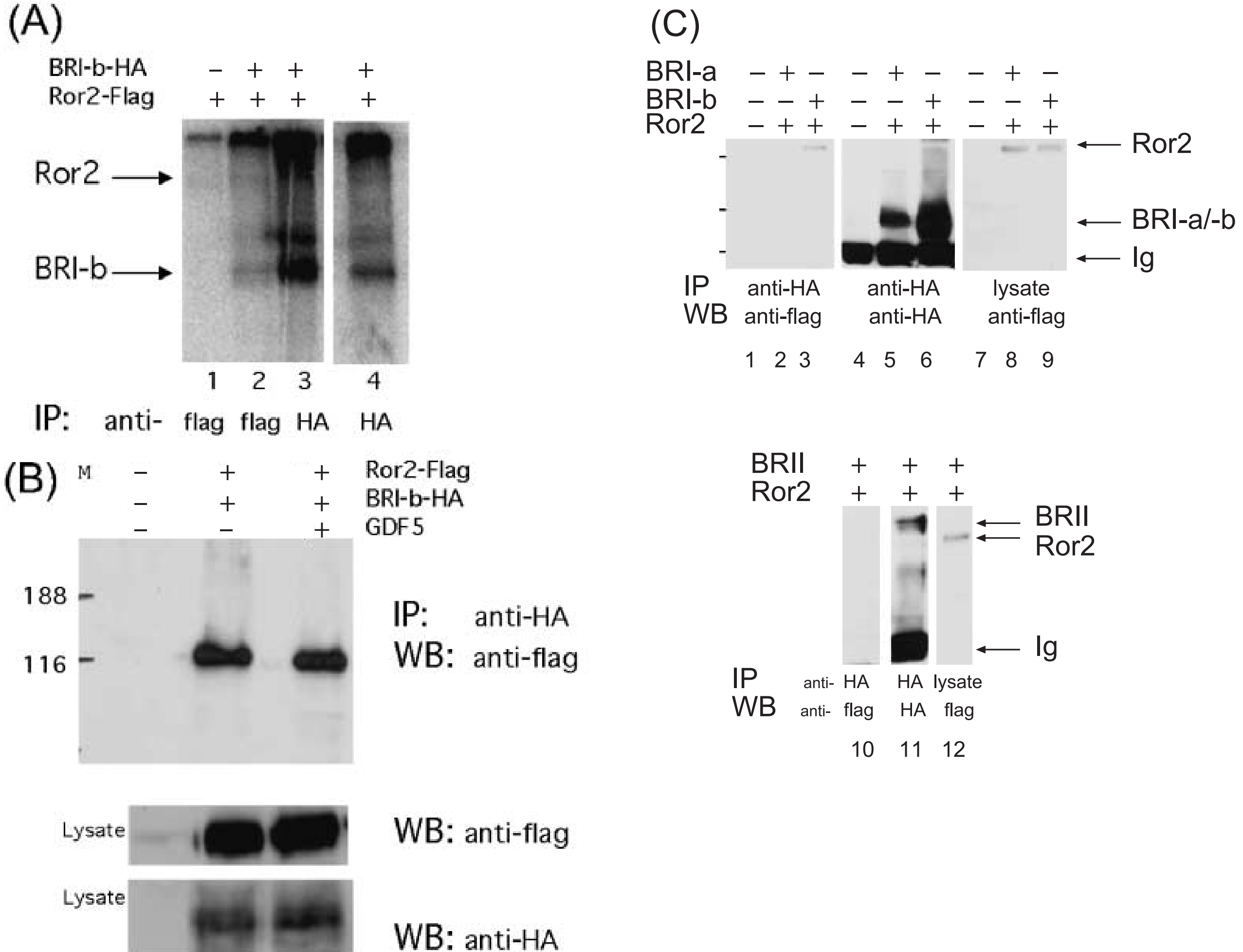

Figure 1 BRI-b and Ror2 interact in the presence and absence of GDF5. (A) COS cells were transfected with Ror2-Flag and BRI-b-HA. After binding and chemical cross-linking of 5 nM ^{125}I-GDF5, immunoprecipitation was performed with either anti-Flag or anti-HA antibodies (as indicated). The position of the ligand-bound receptors is indicated by arrow. (B) COS cells were transfected with Ror2-Flag and BRI-b-HA. Two days after transfection cells were treated with 20 nM GDF5 for 30 min or left untreated. Cell lysates were subjected to immunoprecipitation by anti-HA. Western blotting was performed using anti-Flag. Expression controls were performed on a separate gel using anti-Flag (for Ror2) or anti-HA (for BRI-b). MW of Ror2: 120 kDa. (C) COS cells were transfected with Ror2-Flag, BRI-a-HA, BRI-b-HA and BRII-HA as indicated. Cell lysates were either subjected to immunoprecipitation by anti-HA (lanes 1-6, 10, 11) followed by WB with anti-Flag (lanes 1–3, 10) or with anti-HA for control (lanes 4–6, 11), or the cell lysate was loaded directly to be tested by WB with anti-Flag (lanes 7–9, 12). MW of Ror2: 120 kDa, MW of BRI-a or BRI-b: 70 kDa, MW of BRII: 150 kDa.

COS cells shows that Ror2 has autophosphorylation activity (Fig. 2A, lane 2, Fig. 2B, lanes 1 and 2), the kinase deficient mutant Ror2-KA is not phosphorylated (Fig. 2B, lanes 5 and 6). Next we showed that Ror2 also serves as a substrate of BRI-b, when co-immunoprecipitated (Fig. 2A, compare lanes 2 and 3). This transphosphorylation is independent of Ror2 kinase activity as we show by using Ror2-KA, which still is phosphorylated when BRI-b is coexpressed (Fig. 2B, compare lanes 5 and 7). This transphosphorylation is also independent of addition of GDF5 (Fig. 2B).

Ror2 and BRI-b interact via the frizzled like CRD domain

In contrast to the BMP/GDF type I receptors, which carry a relatively small and compact extracellular domain resembling a structure comparable to an open left hand

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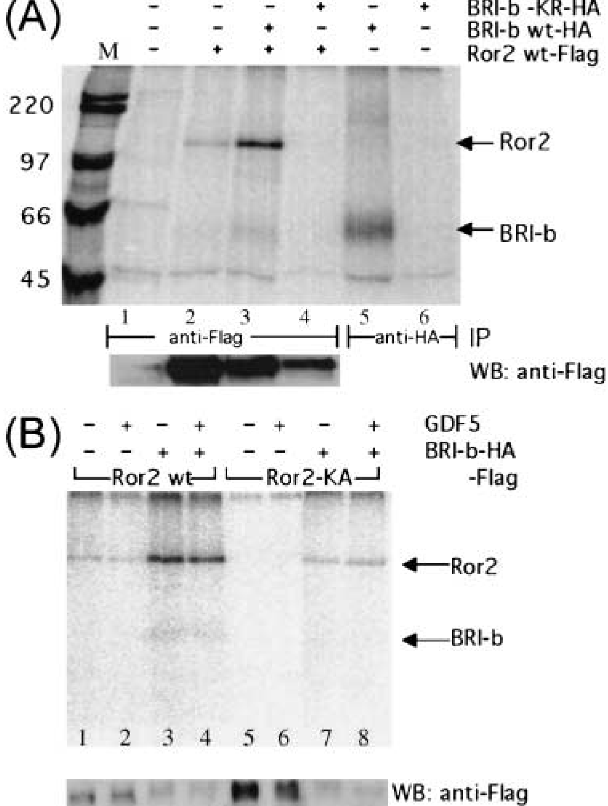

Figure 2 Ror2 is transphosphorylated by BRI-b. COS cells were transfected with indicated receptor combinations: Ror2-wt-Flag (A) in the absence or presence of HA-tagged BRI-b wt or the kinase-deficient mutant BRI-b-KR; in (B) Ror2 wt-Flag or Ror2-KA-Flag in the absence or presence of HA-tagged BRI-b wt. Lysates from same cell numbers were immunoprecipitated with anti-Flag or anti-HA (as indicated) followed by *in vitro* kinase assay as described in Experimental procedures. In (B) cells were treated with 20 nM GDF5 for 30 min (lanes 2, 4, 6, 8) prior to cell lysis. Phosphorylated proteins were visualized by exposure to a phosphor imager screen. Positions of phosphorylated Ror2 and BRI-b are indicated by arrows. For expression control of Ror2 the same amount of immunoprecipitate was run on a separate gel for westernblotting with anti-Flag. Ror2 and BRI-b associate independent of GDF5 addition (A, lane 3; B, lanes 3 and 4); BRI-b transphosphorylates Ror2 within this complex (A, compare lanes 2 and 3; B, compare lanes 1, 2 and lanes 3, 4). This transphosphorylation is independent of Ror2 kinase activity (B, compare lanes 5, 6 and lanes 7, 8) and GDF5 addition (B, lanes 2, 4, 6, 8).

(Kirsch *et al.* 2000), Ror2 has three distinct extracellular domains as illustrated in Fig. 3A. In order to map the site of ligand independent interaction between the serine/threonine kinase receptor BRI-b and the tyrosine kinase receptor Ror2 we generated multiple deletion mutants (Fig. 3A).

First, we deleted each of the extracellular domains separately (Ror2-ΔIg, Ror2-ΔCRD, Ror2-ΔKG) or as a double mutant (Ror2-ΔCRD/KG) and performed immunoprecipitations after coexpression of BRI-b followed by *in vitro* kinase assays. As shown in Fig. 3B deletion of either the Ig- or the KG-domain does not influence the ligand independent association with and transphosphorylation by BRI-b. However, deletion of the frizzled-like CRD region of the receptor results in no interaction with BRI-b. Moreover, an autophosphorylated BRI-b band was co-immunoprecipitated only with the Ror2-wt, Ror2-ΔIg and Ror2-ΔKG (Fig. 3B, lanes 3, 5, 8, 14, 16), whereas this band was absent when Ror2-wt was replaced by Ror2-ΔCRD or Ror2-ΔCRD/KG (Fig. 3B, lanes 10, 12), indicating no association of these mutants with BRI-b. All receptor mutants as well as the wt were expressed at the cell surface as determined by FACS analyses (data not shown). As outlined in the lower panels of Fig. 3B, all truncated versions of Ror2 and BRI-b were equally expressed. These results point out that the CRD domain within the extracellular part of Ror2 is required for the interaction with the BRI-b receptor.

A series of deletion mutants within the cytoplasmic part of Ror2 (Ror2-Δ745, -Δ469) demonstrate that this part of the receptor does not carry the main determinant for interaction with BRI-b. Deletion of almost the entire cytoplasmic part of the receptor (Ror2-Δ469) still results in ligand independent association with BRI-b (Fig. 3C).

To assess the stability of Ror2/BRI-b heteromeric complex, we studied the effect of increasing ionic strength on the complex. Salt concentrations of up to 1 M NaCl have no effect on the Ror2/BRI-b complex. However addition of 0.5% SDS destroyed the complex (Supplementary Material, Fig. S1B; data not shown).

BRI-b and Ror2 co-localize at the cell surface of C2C12 cells

Next we have analysed the expression of both receptors at the cell surface of live cells. In C2C12 cells, Ror2 was labelled endogenously by affinity purified polyclonal antiserum. When BRI-b (HA-tagged) was expressed Ror2 co-localizes with BRI-b at the surface of live cells (Fig. 4).

Taken together, BRI-b and Ror2 associate at the cell surface in a ligand independent fashion via the frizzled-like CRD region of Ror2 in a very stable complex, within which BRI-b transphosphorylates Ror2.

Modulation of the Smad pathway by Ror2

The binding of BMPs and GDFs to their receptors results in the phosphorylation and activation of the type

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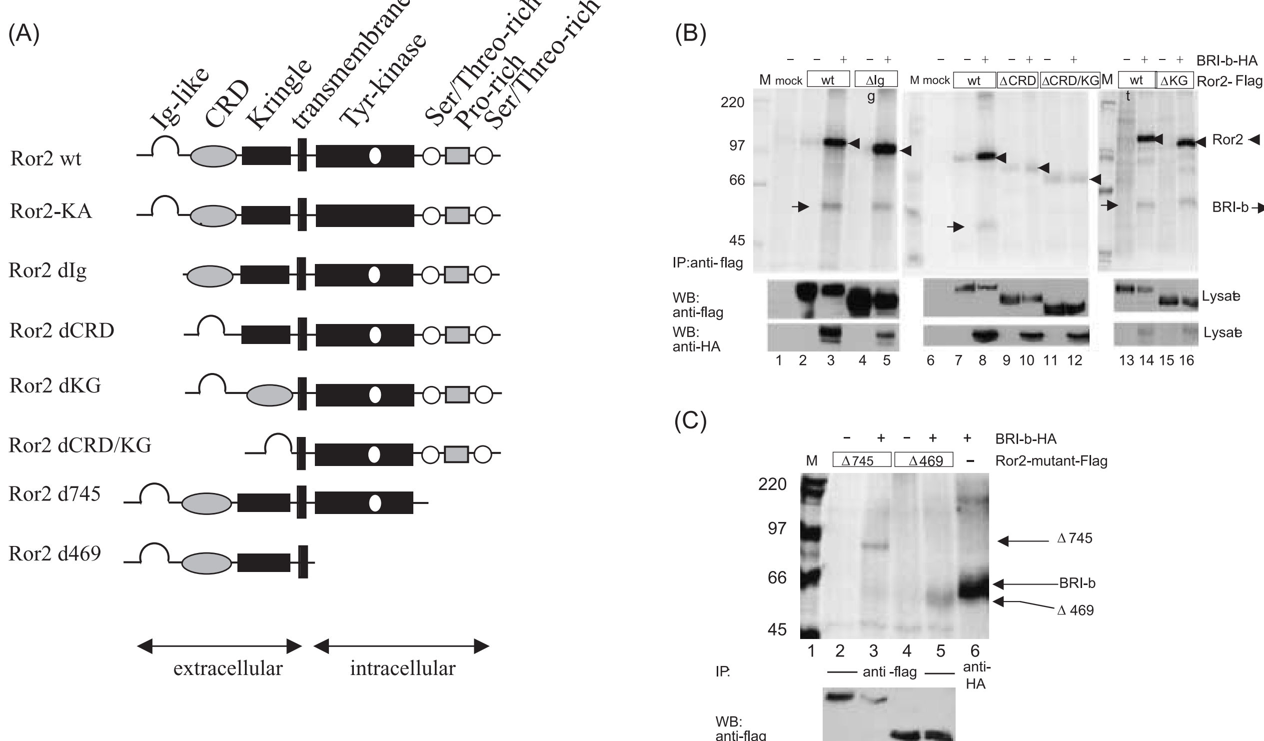

Figure 3 Ror2 associates with BRI-b via its CRD domain. (A) Domain structure of Ror2 and schematic drawing of the different constructs used. (B) Interaction studies with *extracellular* Ror2-mutants: COS cells were transfected with Flag-tagged Ror2 constructs (wt, ΔIg, ΔCRD, ΔCRD/KG) in the presence or absence of BRI-b-HA. Ror2 was immunoprecipitated by anti-Flag antibodies and subjected to *in vitro* kinase assay. The phosphorylated proteins in the Ror2 complex were visualized by phosphoimager. Deletion of CRD disrupts the BRI-b/Ror2 complex (lanes 10 and 12). (C) Interaction studies with *intracellular* Ror2 mutants: COS cells were transfected with Flag-tagged Ror2 constructs (Δ745 and Δ469) in the presence or absence of BRI-b-HA. Experiments have been performed as described in B. Deletions in the cytoplasmic part of Ror2 have no effect on BRI-b/Ror2 complex formation. Expression levels of Ror2 (and deletion mutants) and of BRI-b were determined by anti-Flag and anti-HA immunoblotting (lower panels).

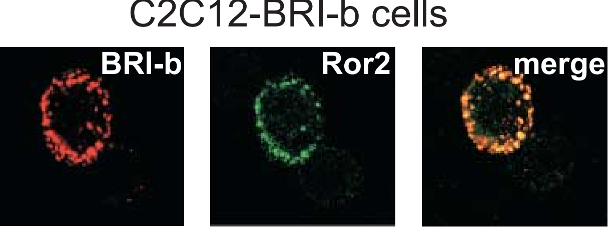

Figure 4 Co-localization of BRI-b and Ror2 in stable C2C12 cells. C2C12 cells stably expressing BRI-b-HA were processed for immunofluorescence co-labelling. After addition of HA-antibody for 1 h at 4 °C the secondary antibody (Cy3-anti-mouse IgG) was applied. The antiserum against the extracellular domain of Ror2 (331/332) was added subsequently followed by Cy2-anti-rabbit IgG. Cells were fixed and analysed by confocal microscopy. Overlays (merge) are also seen.

I receptor and the translocation of Smad1/5/8 to the nucleus where they participate in regulation of gene expression. We therefore tested the effect of Ror2 on Smads in the mouse embryonal carcinoma-derived cell line ATDC5 (Atsumi *et al.* 1990) using a Smad dependent reporter construct (SBE; Jonk *et al.* 1998) (Fig. 5). BRI-b induces the activation of the Smad1/5/8 pathway after addition of GDF5; this induction is dependent on the kinase activity of BRI-b as seen using the kinase-deficient mutant BRI-b-KR. In contrast, Ror2 as well as mutant Ror2 (-KA see Fig. 5 and Ror2-ΔCRD or -Δ745; data not shown) do not induce the Smad pathway. Co-expression of both Ror2 and BRI-b shows significant inhibition of the Smad pathway. These results provide evidence for a modulation of the GDF5/Smad pathway by Ror2. This inhibition is dependent on the kinase activity of Ror2, since coexpression of the kinase-deficient mutant Ror2-KA does not provoke repression of

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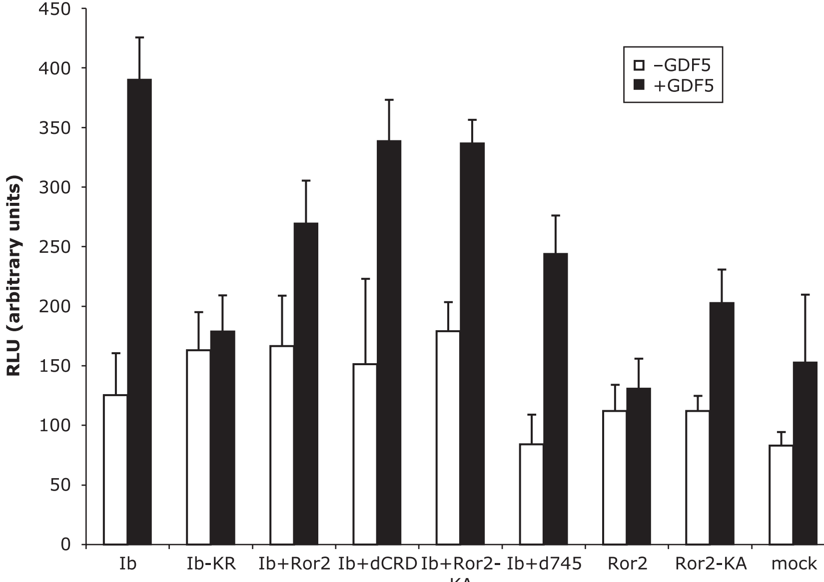

Figure 5 Smad-reportergene assay in ATDC5 cells. ATDC5 cells were transfected with indicated receptor constructs, the reporter gene construct pSBE-luc and for reference pRL-Tk (Dual Luciferase Assay, Promega). Following starvation cells were treated with 5 nM GDF5 (black bars) or left untreated (white bars) for 24 h. Cell lysis and determination of luciferase activity was performed according to the manufacturer's protocol. Standard deviations were calculated from three independent experiments.

Smad-signalling (Fig. 5). Coexpression of Ror2-ΔCRD together with BRI-b also shows no significant Smad-inhibition, presumably due to lack of association of both receptors (see Fig. 3B). Coexpression of the mutant Ror2-Δ745 results in inhibition of BRI-b/Smad signalling due to association with BRI-b (Fig. 3C). In summary these results demonstrate that inhibition of Smad signalling by Ror2 is dependent on both the physical association of both receptors and the kinase activity of Ror2.

GDF5 induces glycosaminoglycan synthesis in ATDC5 cells via the BRI-b

Next we used ATDC5 cells to study the effects of Ror2, BRI-b and GDF5 on chondrogenesis. Chondrogenesis is spontaneously induced in ATDC5 cells after several days in culture due to endogenously produced BMPs (Akiyama *et al.* 2000). The degree of differentiation along the chondrogenic lineage can be quantified by alcian blue staining of cartilage nodules or by measuring the incorporation of [^{35}S]sulphate into glycosaminoglycans which are a major product of chondrocytes and a characteristic component of the cartilaginous extracellular matrix (Shukunami *et al.* 1996; Fujii *et al.* 1999). Furthermore, we confirmed by RT-PCR that GDF5 induces up-regulation of chondrogenic markers such as aggrecan and collagen type II in ATDC5 cells (data not shown). In our experimental setup (i.e. 7 days post-transfection) stimulation of ATDC5 cells with recombinant GDF5 has no additional effect on [^{35}S]sulphate incorporation (Fig. 6-1). As shown in Fig. 6-2, transfection of ATDC5 cells with the BRI-b receptor and treatment of these cells with GDF5 results in a strong increase of glycosaminoglycan production demonstrating the chondrogenic potential of GDF5 via its receptor BRI-b. This effect is dependent on the functional kinase of BRI-b as seen by a kinase deficient receptor (BRI-b-KR), which does not induce glycosaminoglycan production after GDF5 treatment (Fig. 6-3).

Induction of glycosaminoglycan synthesis by Ror2 after treatment with GDF5

Transfection of ATDC5 cells with the Ror2 receptor alone has no effect on [^{35}S]sulphate incorporation (Fig. 6-4). The addition of GDF5 results in a strong increase of glycosaminoglycan production to a level that is even higher than in the BRI-b transfected cells. Treatment with other ligands of the TGF-β/BMP family such as BMP2 or TGF-β2 showed no effect on glycosaminoglycan synthesis in Ror2 transfected cells (data not shown). The induction of glycosaminoglycan synthesis is dependent on Ror2 kinase activity since the kinase deficient mutant Ror2-KA does not respond upon treatment with GDF5 (Fig. 6-5). When compared with the Smad-reportergene assay (Fig. 5), these data suggest that Ror2 does not induce glycosaminoglycan production by directly enhancing Smad activity.

Deletion of the extracellular cysteine-rich domain (ΔCRD) results in a complete loss of glycosaminoglycan synthesis indicating that this site is essential for signal transduction (Fig. 6-7).

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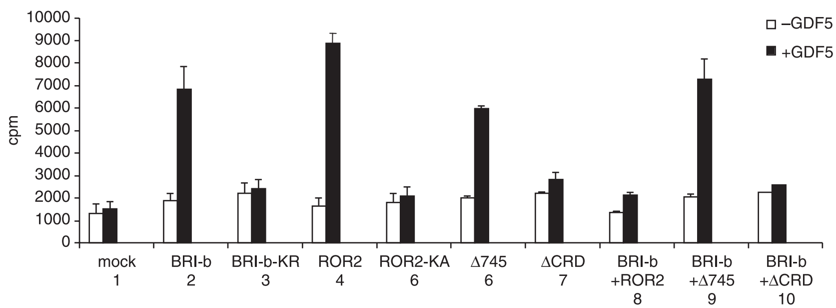

Figure 6 Induction of glycosaminoglycan synthesis in ATDC5 cells. ATDC5 cells were transfected with indicated receptor constructs. Two days after transfection medium was replaced by fresh medium plus 5 nM GDF5 (black bars) or minus ligand (white bars). [$^{35}SO_4$] incorporation was determined as described. Error bars represent SD from 3 to 8 independent experiments. ATDC5 cells were transfected with BRI-b-wt or kinase-deficient (BRI-b-KR), with Ror2-wt or kinase deficient (Ror2-KA) or Ror2 mutants (Δ745, ΔCRD). GDF5 induces glycosaminoglycan synthesis via BRI-b (2) and Ror2 (4); this induction is dependent on their kinase activity as shown in (3) and (5). Co-transfection of both receptors results in the inhibition of GDF5 induced $^{35}SO_4$ incorporation (8). The truncated Ror2-ΔCRD failed to induce chondrogenesis (7), Ror2-Δ745 shows reduced synthesis of glycosaminoglycans (6). Deletion of the tail in Ror2-Δ745 results in loss of inhibition when coexpressed with BRI-b (9). Deletion of CRD in Ror2-ΔCRD results in arrest of the signalling pathway due to loss of Wnt binding (Hikasa *et al.* 2002; Oishi *et al.* 2003) even in the presence of BRI-b (10; and see Discussion). For a schematic drawing of the different Ror2 constructs see Fig. 3A.

Inhibition of chondrogenesis by coexpressed Ror2 and BRI-b

Based on the finding that Ror2 and BRI-b form complexes independent of ligand, we asked the question if Ror2 and BRI-b can modulate their respective pathways. To test this hypothesis, we co-transfected ATDC5 cells with Ror2 and BRI-b and treated them with GDF5. As shown in Fig. 6-8, expression of both receptors resulted in [^{35}S]sulphate incorporation similar to control levels indicating that the receptors inhibit each other. This inhibition might be explained by the direct interaction of the receptors as shown in Figs 1–3.

Coexpression of the mutant Ror2-ΔCRD with BRI-b, however, also results in inhibition of chondrogenesis. As shown in Fig. 3B the mutant Ror2-ΔCRD does not interact with BRI-b, suggesting that BRI-b pathway should not be affected. Due to deletion of the Wnt binding site in Ror2 (Oishi *et al.* 2003), the pathway leading towards glycosaminoglycan synthesis could not be completed. This also suggests that both pathways are necessary for the induction of chondrogenesis, the Smad pathway initiated by BRI-b and the Ror-pathway initiated after ligand binding.

Truncation of the C-terminal part of Ror2 (Ror2-Δ745) causes Brachydactyly BDB in humans (Schwabe *et al.* 2000). When we tested Ror2-Δ745 in ATDC5 cells there was a reduction of GDF5 mediated chondrogenesis seen as compared to the Ror2-wt (Fig. 6-6 and 6-4). When coexpressed with BRI-b there was no inhibition seen as with the wild-type Ror2 (Fig. 6-9 and 6-8). This suggests that the tail region of Ror2 is important for the inhibition of GDF5 mediated glycosaminoglycan synthesis.

Ror2, GDF5$^{bp\text{-}J}$, and BRI-b double mutants indicate interaction *in vivo*

To test for a possible genetic interaction of Ror2 with GDF5 we crossed Ror2$^{+/-}$ mice with GDF5$^{bp\text{-}J+/-}$ and with BRI-b$^{+/-}$ mice. GDF5$^{bp\text{-}J-/-}$ mice have hypoplastic middle and proximal phalanges and a shortening of the humerus and femur whereas GDF5$^{bp\text{-}J+/-}$ mice are completely normal. Ror2$^{-/-}$ mice have a short humerus similar to GDF5$^{bp\text{-}J-/-}$ mice. The phalanges and metacarpals, however, are only mildly affected. Ror2$^{+/-}$ mice are normal. BRI-b$^{-/-}$ mice have hypoplastic middle and proximal phalanges but stylopod and zeugopod are of normal length. The results of the skeletal preparations and the measurement of bone length are shown in Fig. 7. When Ror2 mice were crossed to homozygosity, the inactivation of one GDF5 allele (Ror2$^{-/-}$; Gdf5$^{+/-}$) resulted in the formation of an additional digit between digits II and III. This additional digit consisted of a single phalanx with a distal ossification centre, a characteristic feature of terminal phalanges. We observed the additional phalanx in 100% of the hindlimbs and in 70% of the forelimbs (data not shown). The inactivation of one GDF5 allele in Ror2$^{-/-}$ mice resulted in further shortening of the stylopod. Likewise, inactivation of one Ror2 allele in GDF5 homozygous mice (GDF5$^{bp\text{-}J-/-}$; Ror2$^{+/-}$) results in further shortening, when compared to GDF5$^{bp\text{-}J-/-}$ mice. Double homozygous mice (GDF5$^{bp\text{-}J-/-}$; Ror2$^{-/-}$) have

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Genotype	Wt	Gdf5 –/– Ror2 +/+	Gdf5 –/– Ror2 +/–	Gdf5 +/+ Ror2 –/–	Gdf5 +/– Ror2 –/–	Gdf5 –/– Ror2 –/–	BRI-b –/– Ror2 +/+	BRI-b –/– Ror2 –/–
% length of humerus	100 ± 1.9	84.9 ± 2.2	71.8 ± 2.2	78.7 ± 3.2	73.5 ± 1.7	40.8 ± 1.2	97.3 ± 2.0	63.1 ± 1.2

Figure 7 Ror2, GDF5 and BRI-b show genetic interaction *in vivo*. Mutants for Ror2, Gdf5 and BRI-b were crossed to the indicated allelic combinations. Lengths of mutant humeri are given in percent of the length of the wt humerus. Note the step-wise reduction in size when one or two alleles of Gdf5 are removed on the Ror2$^{-/-}$ background and vice versa. Double homozygous mutants for Ror2 and BRI-b show a drastic decrease in size, which is not part of the BRI-b$^{-/-}$ phenotype and is markedly stronger than the Ror2$^{-/-}$ phenotype alone.

very short limbs with almost absent metacarpals/phalanges and a lack of ossification in the humerus/femur. In these bones, chondrocytes show a complete block in differentiation as demonstrated by H&E histology and the absence of differentiation markers such as Ihh or collagen type X (not shown). In the humerus, there is some ossification but this occurs from the outside at the tuberositas humeri. Double homozygous Ror2/BRI-b mice (Ror2$^{-/-}$; BRI-b$^{-/-}$) had very short limbs with a short humerus/femur. In contrast to the GDF5$^{-/-}$; Ror2$^{-/-}$ mice, histology showed an enlargement of the hypertrophic zone with ossification occurring only from the outside.

Discussion

The brachydactylies are a group of inherited disorders of the hands characterized by shortening of the digits. Mutations in the gene for GDF5 and Ror2 and most recently in BRI-b have been shown to cause distinct subtypes of Brachydactyly (Polinkovsky *et al*. 1997; Oldridge *et al*. 2000; Schwabe *et al*. 2000; Everman *et al*. 2002; Lehmann *et al*. 2003). Several lines of evidence suggested a role for Ror2, BRI-b and GDF5 in chondrocyte differentiation and joint formation. GDF5 mutant mice, as the brachypodism mutant (GDF5$^{bp\text{-}J}$), are characterized by two major phenotypes: aplasia/hypoplasia of the phalanges and phalangeal joints and shortening of the appendicular skeleton with most pronounced effects in the proximal long bones (Storm *et al*. 1994). A reciprocal phenotype of an increase in bone length has been reported following over-expression of GDF5 in the chick limb (Francis-West *et al*. 1999). In the context of the endogenous expression profile this has been interpreted to suggest two roles for GDF5. First, it controls the size of skeletal components in the distal part of the limb by promoting cell adhesion in prechondrogenic precursors. Later, when its expression is restricted to the joints, it signals to the growth plates to control chondrocyte proliferation and differentiation. BRI-b is expressed during early condensation as well as in later stages in growth plate chondrocytes (Zou *et al*. 1997; Baur *et al*. 2000; Yi *et al*. 2000). BRI-b$^{-/-}$ mice have hypoplastic/aplastic phalanges but their long bones are normal. Thus, GDF5 signals mesenchymal condensation through BRI-b. However, for the regulation of differentiation and proliferation of growth plate chondrocytes other receptors, possibly in conjunction with BRI-b, are required. Based on the data presented here we propose that Ror2 can serve as an additional receptor to control cartilage growth.

Since genetic evidences and coherent expression profiles of GDF5 and the receptors BRI-b and Ror2 (Dewulf *et al*. 1995; DeChiara *et al*. 2000; Al-Shawi *et al*. 2001; Matsuda *et al*. 2001; own studies) suggested a functional interplay, we performed biochemical, functional and genetic studies to test this hypothesis. With a series of complementary experiments we show that Ror2 and BRI-b physically interact in the absence and presence of GDF5 (Figs 1–4). Inside this complex BRI-b transphosphorylates Ror2. Using a series of extracellular and intracellular deletion mutants we identified the site of interaction as the frizzled like CRD region (Fig. 3). This domain has been implicated before as the site for Wnt-binding (Oishi *et al*. 2003). The interaction of both receptors, which has been analysed in Figs 1–3 was confirmed on live cells by immunofluorescence co-localization (Fig. 4).

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To investigate the functional impact of the interaction of Ror2 and BRI-b we have used the mouse teratocarcinoma cell line (ATDC5), which represents a well established cell system to study chondrogenesis (Nakamura *et al.* 1999). GDF5 induces the activation of Smad1/5 via BRI-b (Fig. 5) (Nishitoh *et al.* 1996). The Smad pathway is known to be integrated into a complex signalling network with crosstalks to many other signalling cascades such as the MAPK pathway (Lutz & Knaus 2002). In addition, detailed *in vitro* studies have shown that chondrogenic differentiation may be transmitted by Smad-dependent and Smad-independent mechanisms (Fujii *et al.* 1999; Gilboa *et al.* 2000; Nohe *et al.* 2002). Expression of Ror2 in ATDC5 cells does not lead to GDF5-mediated induction of the Smad pathway, but coexpression of both Ror2 and BRI-b results in significant inhibition of Smad activity. This inhibition requires both the association of both receptors as well as the kinase activity of Ror2. Ror2 mutants that fail to interact with BRI-b such as Ror2-ΔCRD show no inhibition of BRI-b induced Smad-signalling (Fig. 5). While ligand binding to plasma membrane receptors and the initiation of signalling molecules has been investigated in great detail, little is known in the cellular compartment responsible for either initiating or selecting the particular pathway that mediates the response. Ror2 might upon association with BRI-b guide this receptor into a membrane compartment, where Smad signalling is abolished.

GDF5 induces the production of glycosaminoglycans in ATDC5 cells, a hallmark for chondrogenic differentiation. This is mediated by BRI-b but, as we show here, also by Ror2 (Fig. 6). Since Ror2 is inactive on the Smad-pathway the effect on glycosaminoglycan synthesis relates to a Smad-independent pathway initiated by the tyrosine kinase receptor Ror2. This pathway is dependent on a functional kinase as shown in Fig. 6.

Inhibition of GDF5/BRI-b mediated chondrogenic differentiation by Ror2 requires the C-terminal portion, but not the CRD region of Ror2. As recently shown by Matsuda *et al.* (2003) the melanoma-associated-antigen (MAGE)-family protein Dlxin-1 associates with this C-terminal tail region of Ror2 and is therefore sequestered at the plasmamembrane. This affects the transcriptional activity of Msx2, a BMP regulated transcriptional repressor. Interestingly, this tail region of Ror2 is missing in patients with BDB.

To explore the interaction of BRI-b, GDF5, and Ror2 *in vivo* we crossed $Ror2^{+/-}$-with $BRI\text{-}b^{+/-}$-mice and $Ror2^{+/-}$ with mice homozygous for the $brachypodism^{J}$ ($GDF5^{bp\text{-}J}$) mutation, which encodes a defective GDF5 allele (Storm *et al.* 1994). The results confirm an epistatic effect between the receptors and the ligand GDF5 since the inactivation of one Ror2 allele in $Gdf5^{bp\text{-}J-/-}$ mice, as well as inactivation of one GDF5 allele or two BRI-b alleles in $Ror2^{-/-}$ mice results in a significant reduction in bone length, a phenotype which is not present in the single mutants. The increase in severity by reducing single alleles demonstrates that the GDF5 and Ror2 pathways are subject to mutation dosage effects and that these two pathways interact. In the double homozygotes ($GDF5^{bp\text{-}J-/-}$; $Ror2^{-/-}$) bone length is reduced even further and chondrocyte differentiation is completely blocked resulting in a purely cartilaginous femur and humerus, a phenotype which is not part of either of the single homozygous mutants. In addition to their crucial role in skeletal development, vertebrate BMPs play roles in multiple other developmental processes (Hogan 1996). To ensure such multiplicity of functions, BMP signalling is tightly regulated during various stages of embryonic development. One emerging conclusion is that this regulation is accomplished by many negative feedback loops that modulate the activity of essential components of the BMP signalling pathway both at the extracellular and intracellular levels (reviewed in Massague & Chen 2000; Miyazono *et al.* 2001). Our studies demonstrate a novel mechanism for the modulation of GDF5 signalling via a tyrosine kinase receptor, Ror2. We show here that BRI-b and Ror2, two different kinase receptors, interact physically and functionally.

Experimental procedures

Constructs

The structure of Ror2 and the mutant construct used in this study are shown in Fig. 3A. The kinase deficient Ror2 (Ror2-KA), and the truncated Ror2-constructs ΔIg, ΔCRD, ΔKG, ΔCRD/KG, Δ469 and Δ745 were generated by site directed mutagenesis using the Gene Editor Kit (Promega) according to the manufacturer's recommendations. To produce the Ror2-KA mutant, the highly conserved lysine residue at position 507 was replaced by alanine. In the truncated constructs, *Xho*I restriction sites were introduced at the positions indicated which were then used to link the flag-tag to the coding sequence. The BRI-b-HA construct was described by us in (Nohe *et al.* 2002). The BRI-b-KR-HA construct was obtained from S. Souchelnytskyi, Uppsala.

Binding and crosslinking

GDF5 was labelled by ^{125}I using the Chloramin T method as described by us earlier (Nohe *et al.* 2002). Confluent 10 cm plates of transfected COS cells were incubated for 4 h at 4 °C with 5 nM ^{125}I-GDF5 in KRH buffer containing 0.5% fatty acid-free BSA. Crosslinking was performed with disuccinimidyl suberate (DSS). Cell lysis and immunoprecipitation was performed as described below.

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Immunoprecipitation, Western blotting, *in vitro* kinase assay

COS cells were transfected with indicated receptor constructs. HA-tagged BRI-b was immunoprecipitated from cell lysates as described (Nohe *et al.* 2002); coexpressed flag-tagged Ror2 was detected after Western blotting using flag-antisera.

For *in vitro* kinase assay, transfected COS cells were harvested and washed twice with ice cold TBS. Cells were lysed in TBS lysis buffer containing 2%NP-40, 20 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM NaF, 10 mM Na_3VO_4 and a mixture of protease inhibitors (Complete-Hoffmann La Roche) for 30 min. Insoluble material was removed by centrifugation. Ror2 was precipitated by specific antibodies for 2 h and the complexes were captured by protein A sepharose. The immunoprecipitates were washed with ice cold lysis buffer with 0.1% detergent and subjected to *in vitro* kinase assay as follows: immunoprecipitates were resuspended in 50 μL of kinase buffer containing 50 mM HEPES pH 7.5, 10 mM $MnCl_2$, 5 mM $MgCl_2$ and 10 μCi of (γ-^{32}P)ATP (300 Ci/mmol, Amersham). The reaction was performed for 20min at RT, washed twice with wash buffer containing 20 mM Tris/HCl pH 8.0, 150 mM NaCl, 20 mM EDTA and 0.1% NP-40 and terminated by adding SDS sample buffer with 5% β-mercaptoethanol. Samples were boiled for 5min and subjected to SDS-PAGE. Phosphorylated proteins were visualized by autoradiography using Phosphoimager.

Immunofluorescence studies on stable C2C12-BRI-b-HA cells

The method was described by us previously on Cos7 cells (Gilboa *et al.* 2000). Here we plated 2×10^4 cells on a chamber slide. Twenty-four hours later we washed cells twice with cold Hanks' balanced salt solution with 20 mM HEPES containing 1% fatty acid-free BSA. This was followed by successive incubations (4 °C, 1 h each with three washes between incubations; all performed in the cold to enable exclusive cell-surface labelling by the antibodies and to avoid internalization) with the following: (1) anti-HA (Roche; 1 : 100), (2) Cy3-anti-mouse IgG (1 : 200), (3) anti-Ror2 (331/332, 1 : 100) and (4) Cy2-anti-rabbit IgG (1 : 200). After washing the cells were fixed in methanol (5 min, –20 °C) and acetone (2 min, –20 °C) and mounted with glycerine gelatine. Fluorescence images were acquired with confocal microscopy.

Generation of stable C2C12-BRI-b-HA cells

Cells were transduced by infection with helper-free VSV-G pseudotyped retroviruses as described (Pear *et al.* 1993). Briefly, 293T cells were co-transfected with the retroviral construct and plasmids for gag-pol and VSV-G. 24 h post-transfection, cells were treated with 10 mM sodium-butyrate for 10 h. Infection of the target cells was performed 48 h and 72 h after transfection. Since the retroviral sequences contain the *gfp*-gene, infected cells could be selected by FACS-sorting.

Smad-reporter gene assay

To determine the GDF5 induced activation of the Smad pathway, ATDC5 cells were co-transfected with the indicated receptor constructs and the luciferase reporter gene construct pSBE-luc (Jonk *et al.* 1998) and pRL-TK for reference. Luciferase activity was measured using a dual luciferase assay system (Promega).

Chondrogenic differentiation assay: incorporation of ^{35}S-sulphate into glycosaminoglycans

Chondrogenic differentiation of ATDC5 cells was determined by measuring the incorporation of [^{35}S]-sulphate into glycosaminoglycans as described (Fujii *et al.* 1999). Briefly, cells were transfected with the indicated receptor constructs using LipofectAMINE™ (Life Technologies) and incubated with 5 nM GDF5 or left untreated. Mock transfected cells represent ATDC5 cells transfected with empty vector DNA. On day 7 post-transfection, cells were labelled with $Na_2{}^{35}SO_4$ (10 μCi/well of a 24-well plate) for 4 h. The radioactive medium was removed and cells were incubated with 6 M guanidine hydrochloride overnight at 4 °C. The lysate was then precipitated with alcian blue and applied on filter by a Skatron. Radioactivity was determined by a Raytest-Counter. SD represent the results of 3–8 independent experiments. The expression level of the constructs used for transfection were tested separately by transient transfection of COS cells followed by Western blot against the epitope tags of the receptors. All DNAs used showed the same expression level.

Mice

Brachypodism (bp) (GDF5$^{bp\text{-}J+/-}$) mice were obtained from Jackson Laboratories. Genotyping was performed using the primers mGdf5 Seq F1: 5′-CGACAGGTCCACGAGAAAGC-3′, and mGdf5 *Seq*RI: 5′-GGT GCG ATG ATC CAG TCG TC-3′. The products were analysed by sequencing or by SSCP analysis. Genotyping of Ror2$^{+/-}$ mice was performed using the primers mRor2-wt: 5′-CCTACTATAGACTCTGATCCTTCTGCC-3′, mRor2-ko-neo1500: 5′-ATCGCCTTCTATCGCCTTCTT-GACGAG-3′, mRor2-ext-4: 5′-CTTAACTGTTCTAGGT-CAAGTATG-3. BRI-b$^{+/-}$ mice were kindly provided by K. Lyons and genotyped as described (Yi *et al.* 2000). Skeletal preparations, histology, staining, and *in situ* hybridization was performed as previously described (Stricker *et al.* 2002)

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Supplementary material

The following material is available from: http://www.blackwellpublishing.com/products/journals/suppmat/GTC/GTC799/GTC799sm.htm: **Figure S1** Ror2 association with BRI-b is resistant to high ionic stringency and DTT but sensitive to SDS. COS cells were co-transfected with Ror2-Flag and BRI-b-HA constructs. (A) Two days after transfection, cells were lysed in lysis buffer containing 150 mM NaCl (lane 1 and 2), 300 mM (lane 3) and 1 M NaCl (lane 4) or with lysis buffer containing 0.1% SDS (lane 5) and 0.5% SDS (lane 6) or solubilized in RIPA buffer (lane 7). (B) Cells were lysed in normal lysis buffer supplemented with or without DTT. Ror2 was immunoprecipitated by anti-Flag antibodies and subjected to *in vitro* kinase assay. The immunoprecipitated proteins were separated on 7.5% SDS-PAGE and the phosphorylated Ror2 proteins and the co-immunoprecipitated phospho-BRI-Ib were detected by Phosphoimager. The expression of Ror2-Flag and BRI-b HA was confirmed by immunoblotting with anti-Flag or anti-HA, respectively, as documented in the lower panels.

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