The mutation ROR2\textsuperscript{W749X}, linked to human BDB, is a recessive mutation in the mouse, causing brachydactyly, mediating patterning of joints and modeling recessive Robinow syndrome

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Mutations in ROR2 result in a spectrum of genetic disorders in humans that are classified, depending on the nature of the mutation and the clinical phenotype, as either autosomal dominant brachydactyly type B (BDB, MIM 113000) or recessive Robinow syndrome (RRS, MIM 268310). In an attempt to model BDB in the mouse, the mutation W749X was engineered into the mouse Ror2 gene. In contrast to the human situation, mice heterozygous for Ror2\textsuperscript{W749FLAG} are normal and do not develop brachydactyly, whereas homozygous mice exhibit features resembling RRS. Furthermore, both Ror2\textsuperscript{W749FLAG/W749FLAG} and a previously engineered mutant, Ror2\textsuperscript{TMlacZ/TMlacZ}, lack the P2/P3 joint. Absence of Gdf5 expression at the corresponding interzone suggests that the defect is in specification of the joint. As this phenotype is absent in mice lacking the entire Ror2 gene, it appears that specification of the P2/P3 joint is affected by Ror2 activity. Finally, Ror2\textsuperscript{W749FLAG/W749FLAG} mice survive to adulthood and exhibit phenotypes (altered body composition, reduced male fertility) not observed in Ror2 knockout mice, presumably due to the perinatal lethality of the latter. Therefore, Ror2\textsuperscript{W749FLAG/W749FLAG} mice represent a postnatal model for RRS, provide insight into the mechanism of joint specification, and uncover novel roles of Ror2 in the mouse.

KEY WORDS: ROR2 receptor, Brachydactyly B, Phalanx formation, Knock-in mouse

INTRODUCTION

Recessive Robinow syndrome (RRS, MIM 268310) and autosomal dominant brachydactyly type B (BDB, MIM 113000) are two distinct syndromes caused by mutations in the ROR2 gene (Afzal and Jeffery, 2003). RRS is characterized by moderately short stature, hemivertebrae, mesomelic limb shortening, brachydactyly, abnormal facial features and genital hypoplasia (Patton and Afzal, 2002). By contrast, BDB is characterized by hypoplasia of the distal and middle phalanges with variable degrees of distal and proximal symphalangism, often accompanied by nail dysplasia (Schwabe et al., 2000).

ROR2 belongs to a small family of receptor tyrosine kinases containing frizzled domains (Forrester, 2002). The extracellular region is characterized by the presence of immunoglobulin (Ig), frizzled-like cysteine-rich (Frz or CRD) and kringle (Kr) domains, all postulated to mediate protein-protein interactions. The intracellular region contains a tyrosine kinase (TK) domain, and a conserved domain consisting of two regions rich in serine and threonine (ST1, ST2) flanking a region rich in proline (PR). Based on the homology of its Frz domain to the Wnt receptors, the Frizzleds, ROR2 was proposed, and then shown, to act as a receptor for Wnts (Masiakowski and Yancopoulos, 1998; Saldanha et al., 1998; Hikasa et al., 2002; Billiard et al., 2005; Oishi et al., 2003; Mikels and Nusse, 2006).

The genetic lesions found in RRS patients consist of homozygous missense, nonsense and frameshift mutations affecting the Frz, Kr and TK domains of ROR2. They are predicted to eliminate or severely reduce receptor function and are thus thought to be loss-of-function mutations (Forrester, 2002). Consistent with this hypothesis, knockouts of Ror2 in the mouse cause a developmental phenotype that displays some of the features of RRS, such as short limbs and brachydactyly, as well as genital, cardiac and craniofacial defects (DeChiara et al., 2000; Takeuchi et al., 2000). By contrast, mutations in ROR2 causing autosomal dominant BDB cluster within two segments immediately upstream and downstream of the TK-encoding region, and are all predicted to result in truncations of the intracellular domain (Schwabe et al., 2000). Because neither homozygous carriers of RRS nor individuals with chromosomal deletions encompassing ROR2 exhibit digit defects (Oldridge et al., 1999), it appears unlikely that the BDB mutations have a loss-of-function effect and it is instead thought that they confer novel gene functions (Forrester, 2002).

In this study, we use a knock-in approach in mice to assess the effect of ROR2\textsuperscript{W749X}, one of the mutations identified in individuals with severe forms of BDB. Unlike Ror2 knockout mice (Takeuchi et al., 2000; DeChiara et al., 2000), Ror2\textsuperscript{W749FLAG/W749FLAG} animals are viable, allowing the study of the effect of this ROR2 truncation postnatally. This animal model exhibits many of the features of RRS, and uncovers novel phenotypes in fertility and body composition. In addition, a differential phenotypic trait, restricted to the digits, was observed between mutants missing the entire Ror2 coding region versus the Ror2\textsuperscript{TMlacZ/TMlacZ} and Ror2\textsuperscript{W749FLAG/W749FLAG} lines. Our observations point to a direct role of ROR2 cytoplasmic mutations in the modulation of joint specification.

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MATERIALS AND METHODS

Generation of knock-in mice, genotyping and RT-PCR assays

Ror2WT749FLAG mice were generated using VelociGene technology (Valenzuela et al., 2003). Briefly, a bacterial artificial chromosome-based targeting vector (BACVEC) was used to replace the sequence coding for domain G750-D930 of mouse ROR2 with the FLAG epitope, followed by a neomycin resistance cassette. ESC transfection, chimera production and genotyping of offspring were as described (Valenzuela et al., 2003). Embryos were genotyped by allele-specific PCR on yolk sac DNA, using the primers: 5'-CTTCCCCAGGCTTCAGTCTCATC-3', 5'-TACCTGTAAGGCCTGGTATTGC-3', 5'-GACTAACAAGGACGATGACGCAA-GC-3' and 5'-TGGATGTGGAATGTGTGCGAG-3'. RNA was isolated with the RNeasy kit (Qiagen, Valencia, CA, USA) from 15.5 days post-coitum (dpc) embryos preserved in RNAlater (Ambion, Austin, TX, USA). First-strand cDNA and PCR amplification were performed as described (Marie et al., 1998), using the primers: 5'-ATTCACTGCTGCCCATCCGC-3', 5'-GATGACGCTGCTGGAGGAAGG-3' and 5'-GTCTGGTGTCATCGTCTTTTGATGC-3'.

Immunoprecipitation and western blotting

Immunoprecipitation followed by protein G-Sepharose (Amersham Biosciences, Piscataway, NJ, USA) precipitation was performed on tissue lysates (1% Brij 96 buffer) with either an anti-FLAG antibody (M2, Sigma-Aldrich, St Louis, MI, USA) or a rabbit anti-ROR2 antibody against the 80 carboxy-terminal amino acids. Immunoprecipitates were separated by SDS/PAGE, immunoblotted with antibodies to ROR2, FLAG or phosphotyrosine (4G10, Millipore, Billerica, MA, USA), and visualized by chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL, USA).

Expression constructs, transient transfection and confocal imaging

ROR2 constructs were generated with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA), cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and transfected into Cos1 cells with ExGene500 (Fer Mentas, Burlington, Canada). Expression constructs were an average of two consecutive scans per animal.

Body composition analysis

Body composition and length were measured on anesthetized mice by dual-emission X-ray absorptiometry (pDEXA Sabre X-Ray Bone Densitometer and software, Norland Medical Systems, Ft. Atkinson, WI, USA). Quality assurance measurements were performed prior to each use. Measurements were an average of two consecutive scans per animal.

Fertility assessment tests

Ror2WT749FLAG and Ror2WT749FLAG littermate males (n=6) were mated with CD-1 virgin females not selected for oestrus. For each trial mating, one female was brought to the male’s cage and separated the following day. Testing continued until each male had been mated with 10 females. The percentages of females presented to each male that exhibited a vaginal plug and that became pregnant were calculated as indexes of plugging rate and pregnancy rate.

Sperm collection, sperm analysis and in vitro fertilization (IVF) assays

The content of the vas deferens and cauda epididymis was released into HTF-0.4% BSA (human tubal fluid, Conception Technologies, San Diego, CA, USA) and analyzed using the Hamilton Thorne Biosciences IVOS System with Animal Motility Software (Beverly, MA, USA). For IVF assays, cumulus-oocyte complexes from superovulated B6C3F1/Crl females were incubated with sperm in 500-μl drops of HTF-BSA at 37°C. Fertilized embryos were transferred 4 to 6 hours later to 75-μl drops of KSOM (Specialty Media, Phillipsburg, NJ, USA) for overnight culture. Two-cell embryos were then transferred, transferred to fresh KSOM and cultured for 72 hours to the expanded-blastocyst stage. Assays were performed on 3-month-old Ror2WT749FLAG/W749FLAG, Ror2WT749FLAG and control littersmates (n=3).

Skeletal analysis and histology

Cleared skeletal preparations were prepared as described (Luftin et al., 1992; Legallais and De Robertis, 1992). Cell proliferation analysis in vivo was carried out by injection of 5-bromo-deoxyuridine (BrDU, Calbiochem, San Diego, CA, USA) at 50 mg/kg 2 hours before euthanasia. For E18.5 and P5 analysis, limbs were formalin fixed and decalcified (Decal Stat High Speed, Decal Corporation, Tallman, NY, USA). Humeri and femurs were then dissected and embedded in Tissue-Tek OCT (Miles, Elkhart, IN, USA). Staining of frozen sections was done with the Dako EnVision+ System and BrDU antibody (Dako, Glostrup, Denmark). Quantitation of BrDU-positive cells was performed with NIH Image (NIH, Bethesda, MD, USA) on six sections from two mice. For E12.5 analysis, limb sections were stained with the BrDU Labeling and Detection Kit II (Roche Applied Science, Indianapolis, IN, USA) and counterstained with DAPI to visualize nuclei. Proliferation rates were calculated from four sections, as the number of BrDU-positive nuclei relative to the total number of nuclei. For prepubertal testes histology, 10-μm frozen sections were stained with Hematoxylin. All other histology was performed on paraffin-embedded tissues by Charles River Laboratories (Wilmington, MA, USA).

In situ hybridization

In situ hybridization was performed on 7-μm paraffin sections of limbs, using digoxigenin-labeled riboprobes prepared as described (Stricker et al., 2002). Whole-mount hybridization was performed as described (Albrecht et al., 2002). Photography was with a binocular microscope and camera (Leica, Bensheim, Germany). The riboprobes used were: Sox9 (Healy et al., 1996), Hoxd11, Hoxd12, Hoxd13, Gdf5, Fgf8 and Shh (Albrecht et al., 2002).

X-ray and bone histomorphometric analysis

Radiography was performed post-mortem at 30 kV for 20 seconds (MX 20, Faxitron X-ray, Wheeling, IL, USA). For histomorphometry, mice were injected with 20 mg/kg calcine and 50 mg/kg demeclocycline at an interval of 2, 5 or 7 days (for 3-, 8- and 24-week old mice, respectively), and sacrificed 2 days after the demeclocycline injection. Dissected femurs were fixed in 70% ethanol, dehydrated and embedded undecalcified in methyl methacrylate. Longitudinal 5-μm sections were cut on a Microm microtome (Richard-Allan Scientific, Kalamazoo, MI, USA) and stained with 0.1% Toluidine Blue, pH 6.4. Static parameters of bone turnover were measured in a defined area between 725 μm and 1270 μm from the growth plate, using OsteoMeasure (Osteometrics, Atlanta, GA, USA). Dynamic histomorphometric parameters were measured as described (Gazzero et al., 2005). Terminology and units are as recommended by the ASBMR Histomorphometry Nomenclature Committee (Parfitt et al., 1987).

RESULTS

Engineering mice carrying a targeted truncation of ROR2 at W749

ROR2WT749X, resulting from a 2246G→A transition mapping downstream of the tyrosine kinase domain (Fig. 1A), is one of several heterozygous mutations found in families affected with classical BDB disorder (Oldridge et al., 2000). To investigate the basis for the two different syndromes associated with Ror2 mutations, we generated knock-in mice expressing ROR2 truncated at W749 and tagged at the carboxy terminus with the FLAG epitope (Ror2WT749FLAG; Fig. 1B). Experimental cohorts were genotyped by PCR (Fig. 1C). Transcription of the truncated ROR2-W749FLAG transcript was verified by RT-PCR (Fig. 1D). Expression and tyrosine phosphorylation of the truncated receptor were confirmed by immunoprecipitation from uterus lysates (Fig. 1E). Like ROR2, ROR2-W747FLAG, a mutein very similar to ROR2-W749FLAG, localizes to the membrane (Fig. 1F;G; identical results have been obtained with ROR2-W749FLAG, not shown).
Altered body mass and skeletal defects in Ror2\textsuperscript{W749X} mutant mice

Homozygous mutant mice were born at the expected Mendelian ratio and were viable. Whereas Ror2\textsuperscript{W749X} is transmitted as a dominant mutation in BDB patients, heterozygous Ror2\textsuperscript{W749FLAG/+} mice were normal and did not display brachydactyly. Homozygous mutant mice appeared smaller (Fig. 2B,D) and exhibited a significant decrease in body weight, apparent before weaning (Fig. 2G). Body composition analysis revealed that Ror2\textsuperscript{W749FLAG/W749FLAG} mice had significantly decreased fat mass and increased lean mass relative to their body weight (Fig. 2I). To determine whether the reduced body mass of Ror2\textsuperscript{W749FLAG/W749FLAG} mice was accompanied by alterations in metabolic rate, we measured food intake, activity, energy expenditure, oxygen consumption and carbon dioxide production. No significant differences between mutant and wild-type littermates were found in any metabolic parameter, or in glucose or insulin tolerance tests (not shown).

Homozygous Ror2\textsuperscript{W749FLAG/W749FLAG} had multiple craniofacial defects. They presented with a shorter snout and epiphora, which deteriorated with age and was often associated with entropion (Fig. 3A). Adult homozygous mutants showed significant shortening and broadening of the nasal bones (Fig. 3B,E,F), as well as significant shortening of the mandible, due to reduced length in the anterior region, from the infradentale to menton landmarks (Fig. 3D,H). The intra-orbital width was significantly increased in mutant mice (Fig. 3B,G). In the postcrani al skeleton, all homozygous mice exhibited a reduced longitudinal axis, brachydactyly in the fore and hind paws (detailed analysis below) and, in 5% of the cases, a bifid digit I in the right hind feet. In addition, 25% of the homozygous mutants had one or more tail kinks (Fig. 2B). Body length, but not width, was significantly decreased in adult Ror2\textsuperscript{W749FLAG/W749FLAG} mice (9.5±0.18 cm versus 10.4±0.25 cm, P=0.005). The shortening of the longitudinal axis resulted from hypoplastic vertebral bodies, rather than from a reduced number of vertebrae (Fig. 2D). Tail kinks were caused by hemivertebrae (Fig. 2E, arrows). In addition, fusions of caudal vertebrae were observed in 5% of the mutants (Fig. 2D, arrowhead). Stylopod and zeugopod bones were shorter by 12%-19% in Ror2\textsuperscript{W749FLAG/W749FLAG} mice of weaning age; however, the differences between mutant and controls became less marked as the animal aged (see Table S1 in the supplementary material). In the autopod, most phalanges and metacarpals remained significantly shorter than in controls (up to 27%, P<0.01; see Table S2 in the supplementary material).

The Ror2\textsuperscript{W749FLAG} mutation does not affect bone remodeling in adult mice

ROR2 has been reported to have signaling activity in osteoblastic cells and to promote osteoblastic activity in vitro (Billiard et al., 2005; Liu et al., 2006), whereas Ror2 knockout embryos exhibit delayed ossification of endochondral bones (DeChiara et al., 2000). We were therefore interested in determining whether ROR2-W749FLAG has an effect in the regulation of bone formation. No abnormalities could be detected in bone histomorphometric
parameters in femurs from 8- to 24-week-old Ror2\textsuperscript{W749FLAG/W749FLAG} mice (Fig. 4C), suggesting that Ror2 does not exert a significant action on the regulation of adult bone remodeling. In actively growing femurs from 3-week-old Ror2\textsuperscript{W749FLAG/W749FLAG} mice, a 52% reduction in trabecular bone volume was observed associated with a 57% decrease in the number of trabeculae. There was no change in the number of osteoblasts per perimeter (not shown), or in the osteoblast surface/bone surface. A lower value of the calculated number of osteoblasts/area was due to lower bone area. Dynamic histomorphometry indicated that the mineral apposition rate was lower in the mutants than in controls, suggesting a reduced osteoblastic function. However, the mineralizing surface per bone surface was higher in the mutant femurs, resulting in an elevation of the calculated bone formation rate. In summary, the low bone volume and the high bone formation rate observed in 3-week-old animals are accompanied by normal osteoblast number; they resolve later in life, and may therefore represent a delay in the process of endochondral ossification in mutant animals rather than a defect in osteoblast differentiation and function.

Ror2\textsuperscript{W749FLAG/W749FLAG} male mice display reduced fertility

In trial matings with CD-1 mice, female Ror2\textsuperscript{W749FLAG/W749FLAG} proved fertile, whereas Ror2\textsuperscript{W749FLAG/W749FLAG} males showed decreased fertility, reflected by a 4% pregnancy rate versus 15% for heterozygous controls (Fig. 5A). Ror2\textsuperscript{W749FLAG/W749FLAG} males had a lower combined testicular weight (118±9 mg versus 165±8 mg, *P*=0.002, 2-month-old animals), which was also significant after correction for their reduced body weight (Fig. 5B). Histologically, early puberty Ror2\textsuperscript{W749FLAG/W749FLAG} testes (16 days) showed a decreased density of seminiferous tubules (Fig. 5D). In addition, most tubules had a reduced diameter and

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Table 1. Ror2\textsuperscript{W749FLAG/W749FLAG} spermatozoa are functional

<table>
<thead>
<tr>
<th>Animal*</th>
<th>Plugged superovulated female</th>
<th>Spermatozoa motility (%)</th>
<th>Spermatozoa concentration (millions/ml)</th>
<th>Progressive spermatozoa (%)</th>
<th>IVF rate* (%)</th>
<th>Number of blastocysts/2-cell embryos (%)</th>
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<tr>
<td>B6D2F1</td>
<td>Yes</td>
<td>71</td>
<td>38.1</td>
<td>56</td>
<td>66/83 (79.5%)</td>
<td>64/66 (96.9%)</td>
</tr>
<tr>
<td>WT-1</td>
<td>No</td>
<td>71</td>
<td>29.6</td>
<td>49</td>
<td>23/87 (26.4%)</td>
<td>23/23 (100.0%)</td>
</tr>
<tr>
<td>WT-2</td>
<td>Yes</td>
<td>66</td>
<td>21.0</td>
<td>45</td>
<td>23/83 (27.3%)</td>
<td>22/23 (95.6%)</td>
</tr>
<tr>
<td>WT-3</td>
<td>Yes</td>
<td>73</td>
<td>55.5</td>
<td>54</td>
<td>55/109 (50.4%)</td>
<td>52/55 (94.5%)</td>
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<tr>
<td>KI-1</td>
<td>No</td>
<td>63</td>
<td>22.1</td>
<td>44</td>
<td>63/96 (65.6%)</td>
<td>62/63 (98.4%)</td>
</tr>
<tr>
<td>KI-2</td>
<td>No</td>
<td>74</td>
<td>25.1</td>
<td>58</td>
<td>61/106 (57.5%)</td>
<td>59/61 (96.7%)</td>
</tr>
<tr>
<td>KI-3</td>
<td>No</td>
<td>63</td>
<td>22.3</td>
<td>43</td>
<td>55/81 (67.9%)</td>
<td>53/55 (96.4%)</td>
</tr>
</tbody>
</table>

|\*Three-month-old males, homozygous mutant (KI) and wild-type (WT) littermates. |
|\*Two-cell embryos/fertilized oocytes. |
contained a single basal cell layer (Fig. 5F), as opposed to control testes, where all the tubules had a multilayered seminiferous epithelium. This abnormal histology partially resolved in adulthood, when degenerative tubules devoid of germ cells were seen only in focal areas of the testis (Fig. 5G). Numerous sperm were noted in the epididymis and ductus deferens, and accessory sex glands were normal. Consistent with the decreased testicular weight, the number of sperm cells collected from Ror2<sup>W749FLAG/W749FLAG</sup> testis was decreased (P=0.04; Table 1). However, mutant and control spermatozoa performed equally well in motility and IVF assays (Table 1). Therefore, the Ror2<sup>W749FLAG</sup> mutation results in delayed germ cell differentiation.
during puberty and decreased sperm production in the adult mouse, but does not affect overall sperm function or fertilizing ability.

**Ror2**<sup>W749FLAG/W749FLAG</sup> mice have defects in early chondrogenic condensation and distal joint specification

To analyze the progression of the skeletal phenotype, embryonic skeletal stains were prepared. At E18.5, no abnormalities were observed in the heterozygotes, whereas homozygous embryos displayed defects in the limbs and spine (Fig. 6N,Q,T). We observed delayed ossification of metacarpals, metatarsals and phalanges, and missing P2 (Fig. 6Q,T). Intervertebral discs were shortened, and 20% of the embryos presented one or two misshapen thoracic vertebrae with rib fusion of the associated ribs (Fig. 6N). These defects represent a subset of the abnormalities displayed by **Ror2** knockout embryos [described in detail elsewhere (DeChiara et al., 2000; Schwabe et al., 2004), and shown in Fig. 6L,O,R,U for comparison], and are milder. At E14.5, **Ror2**<sup>W749FLAG/W749FLAG</sup> embryos displayed hypoplastic skeletons, with a reduction in the size of all the anlagens, including the Meckel’s and nasal cartilages (Fig. 6B). Vertebral deformities and tilting were apparent at this stage (Fig. 6E), but, with the exception of impaired segmentation of the digital rays (Fig. 6H), patterning was normal. These abnormalities are reminiscent of those exhibited by **Ror2**<sup>TMlacZ/TMlacZ</sup> E14 embryos (Fig. 6C,F,I), but are not as severe. Because the cartilaginous elements of **Ror2**<sup>W749FLAG/W749FLAG</sup> embryos displayed anatomical defects consistent with those observed in the skeletons of perinatal and adult mice, we conclude that the function mediated by the carboxy-terminal domain of ROR2 is required prior to E14.5, at the time of cartilaginous condensation.

Histological examination of **Ror2**<sup>W749FLAG/W749FLAG</sup> E13.5 embryos revealed a reduced number of cells in the chondrogenic condensations, whereas cell density did not appear to be affected (Fig. 7B,E,F). Cartilage matrix production by the chondrocytes was evaluated histologically using HAB (Hematoxylin, Alcian Blue) or HGF (Hematoxylin, Fast Green, Basic Fuschin), which stain for proteoglycans (blue) and collagen-associated proteoglycans (fuschia), respectively (Ippolito et al., 1983; Tribioli and Lufkin, 1999). There were no differences in the pattern or intensity of the matrix stain in chondrogenic condensations, whereas cell density did not appear to be affected (Fig. 7K-N). Once the cartilage anlagens formed, their program of development proceeded normally in **Ror2**<sup>W749FLAG/W749FLAG</sup> mutants, except for a delay in the onset of developmental hallmarks, such as hypertrophy and vascularization (Fig. 7G,H), which translated into a slight reduction of bone length at birth (Fig. 7J). However, no change could be detected between mutants and controls in the proliferation rates of condensation or growth plate chondrocytes (Fig. 7K-N). These findings, together with the histological observations, indicate that the **Ror2**<sup>W749FLAG/W749FLAG</sup> skeletal phenotype is not caused by an altered proliferation or differentiation of chondrocytes, but rather by an impaired recruitment of mesenchymal cells into the chondrogenic condensation.

**Ror2**<sup>W749FLAG</sup> interferes with specification of the distal digital interzone upstream of Gdf5

An intriguing aspect of the phenotypes associated with the **Ror2** allelic panel is that both **Ror2**<sup>W749FLAG/W749FLAG</sup> and **Ror2**<sup>TMlacZ/TMlacZ</sup> embryos, expressing truncated ROR2, lack phalanges P2. This stands in marked contrast to **Ror2**<sup>–/–</sup> embryos, where all the elements of the digit are formed (Schwabe et al., 2004) (Fig. 6U), indicating that ROR2 function is dispensable for specification of the distal digit joint, yet truncated ROR2 receptors lacking either part or all of the cytoplasmic domain interfere, in homozygosis, with normal
segmentation of the phalangeal rays. To investigate the potential mechanism, in situ hybridization with markers of limb patterning was performed.

Hoxd11-Hoxd13 are essential for normal patterning of the mouse autopod, and targeted disruption of any one of them causes autopod phenotypes reminiscent to Ror2W749FLAG/W749FLAG (Davis and Capecchi, 1994; Davis and Capecchi, 1996). However, Hoxd11, Hoxd12, and Hoxd13 expression is not affected in Ror2W749FLAG/W749FLAG limbs at E12.5, a stage at which phalanges are being specified (see Fig. S1 in the supplementary material). Similarly, the expression patterns of Shh and Fgf8 were not altered in mutant embryos during E11.5-E13.5 (see Fig. S2 in the supplementary material), indicating that the effect of ROR2 truncation on autopod bone length and patterning is independent of the SHH-FOXD signaling pathway and the SHH-FGF feedback loop.

Finally, the expression of Gdf5, an early marker of joint interzones, was examined. E12.5 Ror2W749FLAG/W749FLAG limbs showed the correct pattern of Gdf5 expression expected at this stage; diffuse expression in the inter-digital mesenchyme and more intense expression in the outerzone of condensation and in the presumptive metacarpo-phalangeal joint interzones as the growth of the digital rays advances (Storm and Kingsley, 1996) (Fig. 8A,B). At E13.5, expression of Gdf5 in Ror2W749FLAG/W749FLAG limbs was correctly restricted to the region of the presumptive digit joints but it showed a decreased intensity and domain in the presumptive proximal interphalangeal interzone (Fig. 8D, arrow). At later stages, expression was detected in the proximal interphalangeal interzone, whereas the region of the presumptive distal interzone failed to show Gdf5 expression (Fig. 8E,F; see also Fig. S3 in the supplementary material). Thus, ROR2W749FLAG seems to interfere with the specification of the distal digit interzone and the subsequent formation of the P2/P3 synovial joint.

DISCUSSION

Ror2W749FLAG/W749FLAG mice as a model of recessive Robinow syndrome

Mutations in Ror2 are responsible for two classes of rare genetic disorders in humans, BDB and RRS, which display distinct modes of inheritance (autosomal dominant and recessive, respectively) and are associated with different sets of gene mutations. In this study, we generated mice bearing the W749X mutation in Ror2, which in humans results in a severe form of BDB. Our results show that Ror2−/− mice do not result in dominant brachydactyly in the mouse. Rather it causes abnormalities similar to those of Ror2 knockout mice, without, however, causing the perinatal lethality observed in these lines. In this respect, it provides an animal model most closely resembling RRS.

In contrast to BDB, which only affects the digits, Ror2W749FLAG/W749FLAG mice exhibit abnormalities in the whole skeleton. Similar to RRS patients and Ror2 knockout mice,
Ror2W749FLAG/W749FLAG mice show hemivertebrae, rib fusions, and shortening of the long bones. In the digits, a missing phalanx causes brachydactyly, a feature also described in the Ror2TMlacZ/TMlacZ mice (DeChiara et al., 2000) and found in some RRS families. Furthermore, about 5% of the animals present a duplicated digit I in the right hind limb, a phenotype also observed in 20% of the Ror2TMlacZ mutants that may be related to the bifid thumbs shown by some RRS patients (Oldridge et al., 2000; Schwabe et al., 2004). The craniofacial defects, characterized by midfacial hypoplasia due to shortened nasal and jaw bones, resemble those of Ror2 knockout mice, albeit manifested to a milder degree. Although hypertelorism, a feature of RRS patients and Ror2−/− mice (Schwabe et al., 2004), is not detected in Ror2W749FLAG/W749FLAG, a wider intra-orbital space, and entropion with epiphora and dochitis are observed. It is possible that changes in the orbital bones of Ror2W749FLAG/W749FLAG mice affect the architecture of the naso-lacrimal ducts, resulting in obstruction and dochitis (Meyer, 1993), as is observed in brachycephalic canine and feline breeds. Overall, in mice, the mutation Ror2W749FLAG does not phenocopy the human BDB phenotype associated with this mutation, resulting instead in a mouse model resembling RRS.

The different pattern of inheritance of the W749X mutation in humans compared with in mice may reflect differences in skeletal development between the two species (Wilkie, 2003), or may be attributed to genetic background effects on the expression of the mutation. Indeed, there are examples of distal phalanx brachydactyly in RRS patients (Patton and Afzal, 2002; Balci et al., 1993), and, conversely, of facial hypertelorism, hypoplastic alae nasi, and high nasal bridge and arched palate in some BDB cases (Hamamy et al., 2006). These observations suggest an overlap in the spectra of clinical features caused by BDB and RRS mutations.
in the proximal phalangeal interzone of the forelimbs, revealing reduced expression of Ror2\(^{W749FLAG/W749FLAG}\) and determining the dimensions of the final skeletal development (Atchley, 1930). We do not observe changes in chondrocyte proliferation in the growth plates of the long bones, once they are formed (Dodds, 2004), or after the overexpression of dominant-negative forms of Ror2 (Hartmann and Tabin, 2001; Guo et al., 2004; Tamamura et al., 2005). As Ror2 can interact with several Wnts (Billiard et al., 2005; Mikels and Nusse, 2006; Hamamy et al., 2006; Oishi et al., 2003), it is conceivable that Ror2 cytoplasmic truncations exert an inhibitory effect on Wnt signaling at the developing joint through non-productive interactions with Wnt ligands. Indeed, genetic and biochemical evidence in C. elegans shows that the extracellular domain of CAM-1 (the only C. elegans ROR homolog) is sufficient to antagonize multiple Wnts in a non-cell-autonomous manner, suggesting that CAM-1 buffers Wnt levels through sequestration (Green et al., 2007). The high degree of similarity shared by nematode and mammalian ROR proteins suggests a conserved ability of ROR receptors to modulate Wnt signaling in a cell-membrane-dependent, but kinase-independent, manner.

It has been proposed that during segmentation of the cartilaginous condensations, each new joint is formed at a distance from the previous one by spatial recovery from Noggin-inhibitory signals (Wnt, GDF5, BMPs) that emanate from the previous interzone (Guo et al., 2004). Under this model, the lack of the distal digital joint in Ror2 mutants could be explained by the shorter length of the digital ray condensations, which would not provide enough distance at the distal end to allow concentrations of a joint-inducing activity to recover from the inhibitory field of the proximal joint. However, if this were the mechanism responsible for lack of the P2/P3 joint in the Ror2\(^{W749FLAG/W749FLAG}\) digits, we would also expect Ror2\(^{-/-}\) mice to lack the P2/P3 joint, as their developing digits are as short as those of Ror2\(^{TMlacZ/TMlacZ}\) and Ror2\(^{W749FLAG/W749FLAG}\) mice (Fig. 6H,I,T,U). Yet, Ror2\(^{-/-}\) mice show normal specification of the digital joints. These observations support a direct role of Ror2 cytoplasmic truncations in blockage of formation of the P2/P3 joint.

Ror2\(^{W749FLAG/W749FLAG}\) mice uncover effects of Ror2 on body composition and male fertility

Survival of the Ror2\(^{W749FLAG}\) homozygous mutant mice makes them useful for studying the role of ROR2 in adult bone. Based on studies using cell lines and mouse calvarial primary cultures, it has been
proposed that ROR2 promotes both osteoblast differentiation and the commitment of mesenchymal stem cells to the osteoblastic lineage (Liu et al., 2006). Although at 3 weeks of age Ror2W749FLAG/W749FLAG mice exhibit a reduction in trabecular bone volume, this phenotype is consistent with the delay in embryonic bone development and resolves later in life. Histomorphometric analysis of femoral bones shows that Ror2W749FLAG has no major role in adult bone homeostasis.

The mutation, however, uncovers previously unrecognized effects of ROR2 in other organs. Ror2W749FLAG/W749FLAG mice display a lean phenotype with a normal metabolic rate, insulin tolerance, and adsorption and elimination of glucose. The reduced adiposity of Ror2W749FLAG/W749FLAG male mice is very similar to that of Bmpgmut homozygous mice, which exhibit infertility and dual defects in spermatogenesis: delay in the initiation of germ cell proliferation during early testicular development; and focal tubular degeneration in the mature testes (Zhao et al., 1996). It will be interesting to investigate whether ROR2 and BMP8B act in the same pathway during spermatogenesis.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/9/?id=DC1

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