Inactivation of LAR family phosphatase genes Ptprs and Ptprf causes craniofacial malformations resembling Pierre-Robin sequence

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SUMMARY
Leukocyte antigen related (LAR) family receptor protein tyrosine phosphatases (RPTPs) regulate the fine balance between tyrosine phosphorylation and dephosphorylation that is crucial for cell signaling during development and tissue homeostasis. Here we show that LAR RPTPs are required for normal development of the mandibular and maxillary regions. Approximately half of the mouse embryos lacking both Ptprs (RPTPα) and Ptprf (LAR) exhibit micrognathia (small lower jaw), cleft palate and microglossia/glossoptosis (small and deep tongue), a phenotype closely resembling Pierre-Robin sequence in humans. We show that jaw bone and cartilage patterning occurs aberrantly in LAR family phosphatase-deficient embryos and that the mandibular arch harbors a marked decrease in cell proliferation. Analysis of signal transduction in embryonic tissues and mouse embryonic fibroblast cultures identifies an increase in Bmp-Smad signaling and an abrogation of canonical Wnt signaling associated with loss of the LAR family phosphatases. A reactivation of β-catenin signaling by chemical inhibition of GSK3β successfully resensitizes LAR family phosphatase-deficient cells to Wnt induction, indicating that RPTPs are necessary for normal Wnt/β-catenin pathway activation. Together these results identify LAR RPTPs as important regulators of craniofacial morphogenesis and provide insight into the etiology of Pierre-Robin sequence.

KEY WORDS: LAR phosphatases, Craniofacial development, Bmp, Wnt, Pierre-Robin sequence, Mouse

INTRODUCTION
Pierre-Robin sequence (PRS) is a craniofacial malformation characterized by defects in mandibular development, which result in micrognathia (smaller lower jaw), glossoptosis (backward displacement of the tongue) and cleft palate (OMIM: 261800) (Robin, 1934; Izumi et al., 2012). Ultimately, PRS results in severe respiratory distress and feeding difficulties postnatally (Evans et al., 2011; Mackay, 2011; Izumi et al., 2012). This developmental phenotype can occur concurrent to, or separately from, additional syndromic malformations (Sheffield et al., 1987; Printzlau and Andersen, 2004; Izumi et al., 2012).

In the developing embryo, the mandible (lower jaw and anterior tongue) and the maxillary (upper jaw and palate) are derivatives of the first pharyngeal arch (PA1) (Santagati and Rijli, 2003; Yoshida et al., 2008; Gittton et al., 2010). The pharyngeal arches are segmented structures of the embryonic head composed of a mesodermal core surrounded by neural crest cell (NCC)-derived mesenchyme and encapsulated in ectodermal and endodermal epithelium (Santagati and Rijli, 2003; Minoux and Rijli, 2010). Both the epithelial and mesenchymal components of the arches directly contribute to their development and patterning through bidirectional signaling crosstalk that controls the proliferation, survival and differentiation of NCC-derived mesenchyme (Clouthier et al., 1998; Thomas et al., 1998; Hu and Helms, 1999; Abu-Issa et al., 2002; Abzhanov and Tabin, 2004; Liu et al., 2005; Haworth et al., 2007; Hu and Marcucio, 2009; Reid et al., 2011; Wang et al., 2011).

Mandibular outgrowth from PA1 is a prerequisite for the appropriate morphogenesis of additional structures, including the tongue and palate. Lengthening of the mandible provides the impetus for the developing tongue to lower, thus allowing the palatal shelves to elevate horizontally above the tongue and eventually fuse, separating the oral cavity from the nasopharynx (Ricks et al., 2002). A failure in this series of events leads to PRS, which occurs in 1:8500 to 1:14,000 infants (Benko et al., 2009; Izumi et al., 2012).

The initial outgrowth of PA1 results from the combined activity of sonic hedgehog and canonical Wnt signaling (Yamaguchi et al., 1999; Brault et al., 2001; Jeong et al., 2004; Jin et al., 2011; Lin et al., 2011; Zhang et al., 2011; Sun et al., 2012). Importantly, conditional inactivation of β-catenin in PA1 mesenchyme leads to a severe reduction in mandibular growth, despite normal NCC migration and establishment within PA1 (Brault et al., 2001). In addition, specific loss of β-catenin activity in the mandibular epithelium results in partial reprogramming of the underlying mesenchyme, leading to the formation of a rudimentary lower jaw (Jin et al., 2011; Sun et al., 2012).

Additional pathways required to specify the identity of mesenchymal cells within PA1 include endothelin, fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling (Santagati and Rijli, 2003; Minoux and Rijli, 2010). In particular, loss or gain of BMP pathway activity results in dysregulated mandibular epithelial/mesenchymal signaling, leading to an undersized PA1 and the truncation of the mandibular region (Stottmann et al., 2001; Ishii et al., 2005; Liu et al., 2005; Bonilla-Claudio et al., 2012). BMP signaling is also implicated in the timing of cranial neural crest-derived mandibular bone and cartilage differentiation during craniofacial development (Liu et al., 2005; Merrill et al., 2008; Bonilla-Claudio et al., 2012).

Receptor protein tyrosine phosphatases (RPTPs) of the mammalian leukocyte antigen related (LAR) family comprise three genes: Ptprd (RPTPδ), Ptprs (RPTPα) and Ptprf (LAR) (Chagnon
et al., 2004; Hendriks et al., 2013). LAR family phosphatases have been implicated in several key embryonic signaling pathways, including those involved in the negative regulation of growth factor receptors such as EGFR, RET and MET, and potentially modulate canonical Wnt/β-catenin signaling (Kypa et al., 1996; Müller et al., 1999; Tisi et al., 2000; Wang et al., 2000; Ennslen-Craig and Brady-Kalnay, 2004; Machide et al., 2006; Haapasalo et al., 2007; Uetani et al., 2009; Zheng et al., 2011). Although LAR family phosphatases are expressed in several embryonic and adult tissues (Schapaevld et al., 1998), mice mutant for single family members generally survive to adulthood, reflecting a functional redundancy within the family (Schapaevld et al., 1997; Elchebly et al., 1999; Wallace et al., 1999; Uetani et al., 2000; Thompson et al., 2003; Wang et al., 2011). Accordingly, Ptprs−/+ genetic background. Throughout, control embryos were taken as E0.5. All experiments involving mice were approved by the McGill Animal Care Committee and were performed following the manufacturer’s instructions. All fluorescent stainings were mounted in SlowFade Gold mounting medium (Invitrogen), and images were acquired using a Zeiss AxioPlan 2 microscope or a Zeiss LSM 5 Pascal/Axiovert 200M confocal microscope.

**Cell culture**

MEFs were derived from control and Ptprs−/Ptprd−/Ptprf− triple-knockout embryos at E13.5 by standard procedures. Briefly, embryos were dissected in PBS, the internal organs removed, and the connective tissue trypsinized to a single-cell suspension at 37°C. Trypsinization was stopped with 10% fetal bovine serum (FBS; Wisent) in DMEM medium, and mesenchymal cells were allowed to adhere in 10-cm plates. After 8 hours the plates were washed with PBS to detach non-mesenchymal cells. Cells were maintained in DMEM medium supplemented with 10% FBS and penicillin-streptomycin (Invitrogen) until passage five, when they were used experimentally. For immunofluorescent staining, cells were seeded on acid-washed glass coverslips and allowed to adhere overnight prior to treatment.

**In situ hybridization**

Embryo dissections and processing, as well as whole-mount and section in situ hybridizations, were performed as described (Grote et al., 2008). Probes for Ptprf and Ptprs have been described (Uetani et al., 2009). Probes for Crabp1, Col2a1, Runx2 and Msx1 were generated from E11.5 whole-embryo cDNA using specific primers (supplementary material Table S1). The resultant PCR products were cloned into the pGEM-T Easy vector (Promega) and transcribed following standard procedures using SP6 or T7 RNA polymerase and DIG-labeled dNTPs (Roche).

**Immunohistological staining**

Hematoxylin and Eosin (H&E) staining of control and double-mutant embryos was performed as described (Uetani et al., 2009). For immunohistological stainings, tissues were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight, embedded in OCT compound (Sakura Finetek), and cryosectioned at 12 μm. Alternatively, treated mouse embryonic fibroblasts (MEFs) were fixed in 4% PFA in PBS for 20 minutes at room temperature, and rinsed twice with PBS. Samples were permeabilized with 0.25% Triton X-100 in PBS for 5-10 minutes, blocked with DAKO Protein Block (serum-free) solution for 1 hour at room temperature and incubated with the following antibodies: rabbit anti-phospho-histone H3 (1:400, Millipore), rabbit anti-β-catenin (1:500, Millipore), mouse anti-dephosphorylated β-catenin (1:100, Millipore), rabbit anti-phosphorylated SMAD1/5 (1:50, Cell Signaling Technology) and mouse anti-CRABP1 (1:200, Abcam). Secondary detection employed anti-rabbit or anti-mouse secondary antibodies labeled with Alexa Fluor 488 or Alexa Fluor 568 (1:400, Invitrogen). Samples were counterstained with Alexa Fluor 546-labeled phalloidin (1:400, Invitrogen) and/or DAPI (50 μg/ml, Invitrogen). For apoptotic index, sections were labeled by TUNEL using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instructions. All fluorescent stainings were mounted in SlowFade Gold mounting medium (Invitrogen), and images were acquired using a Zeiss AxioCam MRc dissecting microscope and camera. MEFs were derived from control and Ptprs−/Ptprd−/Ptprf− triple-knockout embryos at E13.5 by standard procedures. Briefly, embryos were dissected in PBS, the internal organs removed, and the connective tissue trypsinized to a single-cell suspension at 37°C. Trypsinization was stopped with 10% fetal bovine serum (FBS; Wisent) in DMEM medium, and mesenchymal cells were allowed to adhere in 10-cm plates. After 8 hours the plates were washed with PBS to detach non-mesenchymal cells. Cells were maintained in DMEM medium supplemented with 10% FBS and penicillin-streptomycin (Invitrogen) until passage five, when they were used experimentally. For immunofluorescent staining, cells were seeded on acid-washed glass coverslips and allowed to adhere overnight prior to treatment. Cells were treated with 100 ng/ml recombinant mouse BMP4 or recombinant human WNT3A for 30 minutes, following which protein was collected or cells were fixed. For experiments involving the GSK inhibitor, cells were treated with 3 μM (2’Z,3’E)-6-bromoindirubin-3'-oxime (BIO; EMD) for 30 minutes prior to fixation or RNA extraction.

**Real-time quantitative PCR**

MEFs were treated with the indicated chemicals, and then rinsed with PBS twice. Protein was extracted on ice using RIPA buffer containing NaF, NaVO₃, and EDTA-free Complete Protease Inhibitors (Roche). Protein concentration was calculated using the Bradford Assay Kit (Bio-Rad), and 30-50 μg protein was run on an SDS-PAGE gel. Protein was transferred to PVDF membranes and blotted by standard methods. Antibody dilution and blocking were as recommended by the manufacturers. Antibodies used were anti-phospho-SMAD1/5 and anti-SMAD1 (Cell Signaling Technology), anti-α-tubulin (Abcam), and HRP-conjugated anti-mouse secondary (Santa Cruz) or HRP-conjugated anti-rabbit secondary (Abcam) antibodies. Signal was detected using the Immobilon Western Chemiluminescent HRP Substrate Kit (Millipore). When necessary, blots were stripped with ReBlot Plus Strong solution (Millipore) prior to reblotting with a different antibody.
Image analysis and statistics
Proliferative and apoptotic indices were determined using the Measure function in the Zeiss AxioVision 4 image acquisition program. Cell counting was performed on six sections spanning the mandibular arch in each of three controls and three affected double-mutant embryos between E10.5 and E12.5 using Adobe Photoshop. Statistical analyses were performed using unpaired two-tailed Student’s t-tests. Data are presented as mean ± s.e.; \( P<0.05 \) was considered significant.

RESULTS
Craniofacial skeletal malformations in \textit{Ptprs}−/−;\textit{Ptprf}−/− embryos
We have previously reported a requirement for the LAR family members \textit{Ptprs} (RPTPs) and \textit{Ptprf} (LAR) in craniofacial morphogenesis (Uetani et al., 2009). In particular, approximately half of the embryos lacking \textit{Ptprs;Ptprf} exhibit micrognathia and cleft palate at E18.5, defects that are not observed in the other genotypes of the allelic series. As these are common craniofacial abnormalities in infants, we undertook a detailed characterization of this phenotype.

To visualize gross malformations of the mouse skeleton we first performed Alizarin Red and Alcian Blue staining, which identifies mineralized bones and cartilage, respectively. An analysis of the torso and limb skeletal elements failed to reveal any significant alterations in the double-mutant embryos (supplementary material Fig. S1A,B). By contrast, evaluation of skull preparations from \textit{Ptprs}−/−;\textit{Ptprf}−/− and control embryos at E18.5 revealed defects in calvarial development, including abnormal frontal and parietal bones, an absence of the nasal capsule, and truncation of the premaxilla, although the maxilla appeared grossly normal (Fig. 1A,B). Furthermore, loss of \textit{Ptprs} and \textit{Ptprf} resulted in dysmorphogenesis of the oral cavity, as double-mutant embryos lack palatine bones, which, together with a dysmorphic basiophenoid and absent pterygoid processes, are consistent with cleft palate (Fig. 1C,D). Strikingly, \textit{Ptprs}−/−;\textit{Ptprf}−/− embryos harbored severe defects in mandibular morphogenesis. In comparison to age-matched littermates, affected DKO mandibles were narrower and shortened, with aberrant bone deposition between the dentary bones (Fig. 1E,F). Additionally, the distal symphysis was fused at the midline in double-mutant mandibles, whereas the rostral processes (angular, coronoid, condylar) were present, suggesting that the proximal mandible was patterned correctly (Fig. 1E,F). The mandibular hypoplasia and cleft palate were clearly visible in E18.5 coronal sections stained with H&E (Fig. 1G,H). These sections additionally revealed that the DKO embryos had a small tongue (microglossia) located further back in the oral cavity (glossostosis) (Fig. 1I,J). This shift was accompanied by a marked reduction in airway space (Fig. 1I,J).

The striking triad of micrognathia, glossostosis and cleft palate in embryos lacking LAR family phosphatases prompted us to more rigorously investigate their association during mandibular development. In accordance with our previous investigation (Uetani et al., 2009), 39% of \textit{Ptprs}−/−;\textit{Ptprf}−/− embryos harbored micrognathia. Notably, all of these embryos also exhibited microglossia and palatal defects, including cleft palate and palatal bone abnormalities at E16.5 and E18.5 (n=7). All micrognathic \textit{Ptprs}−/−;\textit{Ptprf}−/− embryos analyzed also had glossostosis. Conversely, \textit{Ptprs}−/−;\textit{Ptprf}−/− embryos without micrognathia had normal tongues and palates (n=11). In addition, neither controls nor embryos with a single allele of either \textit{Ptprs} or \textit{Ptprf} displayed these defects (n=35), highlighting the strong association between the defects in mandible, tongue and palatal shelf development in the affected double mutants. Together, these defects comprise the morphological features of PRS.

\textit{Ptprs}−/−;\textit{Ptprf}−/− embryos show a patterning defect in cartilage and bone deposition
To investigate the origin of the craniofacial skeletal defects observed in \textit{Ptprs}−/−;\textit{Ptprf}−/− embryos, we characterized the onset of
craniofacial bone ossification. Consistent with the E18.5 skeletal analysis, Alizarin Red and Alcian Blue staining at E14.5 revealed that appendicular bones and cartilages were normal in \textit{Ptprs}\textsuperscript{−/−};\textit{Ptprf}\textsuperscript{−/−} embryos (supplementary material Fig. S1C,D). However, E14.5 DKO embryos were already identifiable as having an undersized lower jaw and lacking a tongue (Fig. 2A,B; data not shown). Cranial bone and cartilage tissues were dysmorphic, including shortened cartilage anlagen of the premaxilla and nasal capsule (Fig. 2C,D). Most strikingly, \textit{Ptprs}\textsuperscript{−/−};\textit{Ptprf}\textsuperscript{−/−} embryos had a dysplastic Meckel’s cartilage, with foreshortened and mispatterned middle processes that were aberrantly fused at the distal tip (Fig. 2E,F), while the proximal arms articulated correctly with the middle ear capsule (supplementary material Fig. S1E,F). Furthermore, mandibular bone deposition occurred medially in double-mutant embryos in comparison with control mandibles, resulting in premature fusion of the dentary bones (Fig. 2E,F). Together, these results identify a role of LAR family phosphatases in the patterning of mandibular cartilage and bone structures.

The inappropriate morphogenesis of Meckel’s cartilage and mandibular bone suggested a possible misspecification of precursor cell populations at earlier time points. Differentiation of mesenchymal cells to chondrocytes and osteoblasts that lay down Meckel’s cartilage and dentary bone initiates at ~E12.5 in the mouse (Ito et al., 2002). Early chondrocytic populations are marked by the expression of collagen 2α1 (Zhao et al., 1997), whereas osteoblast lineages are specified by the transcription factor Runx2 (Funato et al., 2009). To assess the integrity of chondrocyte and osteoblast cell populations in the developing mandibular process, we performed \textit{in situ} hybridization and immunostaining for the mitotic marker phospho-histone H3 (Fig. 3A-K). Specifically, \textit{Ptprs}\textsuperscript{−/−};\textit{Ptprf}\textsuperscript{−/−} embryos had ~70% reduction in pharyngeal arch cell proliferation at E10.5 (Fig. 3A-C), a defect that was largely maintained in the proximal arch of E12.5 DKO embryos, whereas proliferation in the distal mandibular arch more closely resembled control levels (Fig. 3D-G). In contrast to controls, DKO embryos show aberrant specification of the \textit{Col2a1} chondrocyte marker (open arrowheads) in the medial mandibular cartilage at E14.5. Meckel’s cartilage (D,F, black arrowheads) and Meckel’s cartilage precursors (G-J), respectively. Hy, hyoid cartilage; MB, mandibular bone; MC, Meckel’s cartilage; ME, middle ear; NC, nasal capsule; Pr, parietal bone; other labels as Fig. 1.

\textbf{Ptprs;Ptprf deficiency affects mandibular cell proliferation}

To determine the origin of the mandibular patterning defect observed in the double-mutant embryos we first asked whether it was a result of defective cranial NCC migration into the pharyngeal arches. We performed whole-mount \textit{in situ} hybridization and immunofluorescence with the NCC marker CRABP1 at E9.5, which revealed a similar migratory pattern in control and \textit{Ptprs}\textsuperscript{−/−};\textit{Ptprf}\textsuperscript{−/−} embryos (supplementary material Fig. S3A-D). TUNEL staining of migratory NCCs also failed to show any difference in NCC survival (supplementary material Fig. S3C-E). Together, these results argue against a defect in the delamination, migration or survival of cranial NCCs in LAR family-deficient embryos.

We next investigated whether apoptosis and proliferation were affected within the pharyngeal arches at later time points. As observed with NCCs, TUNEL staining of mandibular tissue failed to reveal any significant change in cell survival between mutant and control embryos (Fig. 3A,B; data not shown). By contrast, embryos lacking RPTP-\(\sigma\) and LAR exhibited a marked decrease in proliferation throughout PA1 and the developing oral cavity, as shown by immunostaining for the mitotic marker phospho-histone H3 (Fig. 3A-K). Specifically, \textit{Ptprs}\textsuperscript{−/−};\textit{Ptprf}\textsuperscript{−/−} embryos had ~70% contrast to controls, DKO embryos show aberrant specification of the \textit{Col2a1} chondrocyte marker (open arrowheads) in the medial mandibular arch. (I,J) \textit{In situ} hybridization of coronal sections at E12.5. (G,H) In contrast to controls, DKO embryos show aberrant specification of the \textit{Col2a1} chondrocyte marker (open arrowheads) in the medial mandibular arch. (I,J) Similarly, aberrant specification of the \textit{Runx2} osteoblast marker (black arrowheads) occurs in DKO embryos. Asterisks indicate the dysmorphic mutant version. Dashed lines indicate the lower jaw (E,F) and Meckel’s cartilage precursors (G-J), respectively. Hy, hyoid cartilage; MB, mandibular bone; MC, Meckel’s cartilage; ME, middle ear; NC, nasal capsule; Pr, parietal bone; other labels as Fig. 1.
Ptprf and Ptprs are co-expressed in the distal mandibular process

The developmental defects observed in double-mutant embryos raised the question of where LAR family phosphatases act in the developing mandibular and maxillary processes. To address this we determined the mRNA expression patterns of Ptprf and Ptprs by in situ hybridization throughout early craniofacial development. Interestingly, at E9.5-10.5 Ptprf was strongly expressed in the distal epithelium and mesenchyme of PA1 but expressed at lower levels in the proximal regions (Fig. 4A,B). Conversely, Ptprs was more diffusely expressed throughout the PA1 mesenchyme and was absent from the distal epithelium (Fig. 4D,E). At E12.5, Ptprf was expressed at relatively low levels in the mandibular arch mesenchyme and at higher levels throughout the oral epithelium, whereas Ptprs was expressed at similar levels in both compartments (Fig. 4C,F). This expression continued at E15.5, as Ptprf was expressed in the oral epithelium and palatal seam, which is the site of secondary palatal shelf fusion, whereas Ptprs was only weakly expressed in the seam region at this stage (supplementary material Fig. S4A-D). Hence, the expression patterns of Ptprf and Ptprs align well with a role in patterning the developing pharyngeal arch, and might suggest a later role in palatal shelf morphogenesis.

Wnt and Bmp signaling pathways are dysregulated in Ptprs−/−;Ptprf−/− deficient cells

Much of the cellular behavior within PA1, including survival, proliferation and differentiation, is dictated by reciprocal signaling between the epithelial and mesenchymal compartments (Santagati and Rijli, 2003; Minoux and Rijli, 2010). To identify the molecular alterations caused by Ptprs−/−;Ptprf−/− deficiency, we characterized several signaling pathways involved in pharyngeal arch morphogenesis at E10.5. Given the stronger distal expression of Ptprs and Ptprf in the developing pharyngeal arch, we first asked whether distal epithelial signaling of the BMP pathway was perturbed upon loss of PPTP-σ and LAR. We examined the downstream signaling effector phosphorylated (p) SMAD1/5 by immunofluorescence. This experiment revealed an increase in pSMAD1/5 signal in the distal arch of double mutants, which showed a strong increase in pSMAD1/5 signal in PA1 (Fig. 5A,B). To confirm this, we performed in situ hybridization for the BMP-regulated gene Msx1 on E10.5 transverse sections. In control embryos Msx1 is expressed normally in the medial-distal region of the pharyngeal arch, whereas expression is expanded proximal-laterally upon loss of the LAR family phosphatases (Fig. 5C,D). The specific increase in BMP signaling was further confirmed by RT-qPCR analysis of dissected PA1, which indeed demonstrated an upregulation of Msx1 in DKO embryos, whereas target genes of endothelin 1 and FGF signaling were unchanged (Fig. 5E). Intriguingly, the area of increased BMP signaling preceded the aberrant chondrogenesis and osteogenesis observed in the distal arch.

To confirm the effect of LAR family phosphatase deficiency on BMP signaling we used primary mouse embryonic fibroblasts (MEFs) deficient for Ptprs, Ptprf and the third LAR family member, Ptprd. These triple-knockout (TKO) MEFs were used instead of Ptprs−/−;Ptprf−/− MEFs to avoid functional compensation by RPTP-σ, which is not normally expressed in the mandibular arch (data not shown). In the absence of ligand, a weak activation of pSMAD1/5 was observed in LAR family-deficient cells (Fig. 5F,G). In response to recombinant (r) BMP4 treatment, TKO MEFs showed a marked upregulation and more efficient nuclear localization of pSMAD1/5 as detected by immunofluorescence (Fig. 5H,I). This result was confirmed by western blot analysis, which showed a strong increase in pSMAD1/5 signal in TKO MEFs as compared with wild type, while total SMAD1 levels remained unchanged (Fig. 5J). Furthermore, in vitro analysis of the BMP
signaling response revealed an upregulation of both the chondrocytic genes **Sox9** and **Col2a1** and the osteoblast gene **Runx2** in wild-type fibroblasts upon rBMP4 stimulation (Fig. 5K).

Strikingly, TKO cells were further sensitized to BMP ligand, as they upregulated both chondrocytic and osteoblast markers by 2- to 12-fold over the wild-type fibroblast response (Fig. 5K). These results suggest that LAR family phosphatases dampen BMP signaling in mesenchymal cells and might limit BMP-driven chondrogenesis.

We next analyzed the Wnt/β-catenin response in LAR family phosphatase-deficient embryos. Interestingly, immunofluorescent labeling for transcriptionally active Ser22/37-dephosphorylated β-catenin (Wu and Pan, 2010) revealed a widespread defect in the mandibular arch of DKO embryos at E10.5. In control mandibular arch active β-catenin signal is apparent in the epithelial and mesenchymal compartments of most of the pharyngeal arch, whereas **Ptprs**<sup>−/−</sup>;**Ptprf**<sup>−/−</sup> embryos show a significant reduction of β-catenin activity in both compartments, although total β-catenin protein levels are unchanged (Fig. 6A,B; supplementary material Fig. S5A,B). Similarly, in situ hybridization for the transcriptional target **Axin2** confirmed the more medial activity of canonical Wnt signaling in control pharyngeal arches, whereas double-mutant embryos exhibited a strong reduction in the extent of **Axin2** expression (Fig. 6C,D). The impairment of Wnt/β-catenin signaling was further confirmed by RT-qPCR analysis of dissected PA1 tissue, which revealed strong downregulation of the canonical target genes **Axin2** and **Ccn2** in DKO embryos (Fig. 6E).

In MEF cultures, treatment of control cells with recombinant (r) WNT3A resulted in clear localization of β-catenin to the nucleus (Fig. 6F,H). By contrast, LAR family-deficient MEFs retained β-catenin in the cytoplasm upon stimulation with rWNT3A.
This result was validated with an anti-dephosphorylated Ser22/37 β-catenin antibody that recognizes the active form of β-catenin (Wu and Pan, 2010). Whereas wild-type cells accumulated dephosphorylated nuclear β-catenin, LAR family-deficient cells remained negative for this active form of β-catenin (supplementary material Fig. S5C-F). We next assessed the Axin2 transcriptional response by qPCR. This revealed a severe impairment in Axin2 transcriptional activation upon treatment of TKO MEFs with rWNT3A, in sharp contrast to the strong upregulation observed in control MEFs (Fig. 6L). Hence, LAR family-deficient MEFs are refractory to Wnt signaling. This was further confirmed by lineage marker analysis in which rWNT3A treatment of control cells suppressed Sox9 and Col2a1 transcription and left the osteoblastic marker Runx2 unchanged, whereas loss of LAR family activity specifically abrogated the Sox9/Col2a1 chondrocytic response in MEF culture (Fig. 6M).

To further delineate the level at which the LAR phosphatases act on the Wnt/β-catenin pathway, we used the GSK3β inhibitor BIO to prevent β-catenin targeting for proteosome degradation (Meijer et al., 2003). Upon rWNT3A and BIO treatment, TKO MEFs responded to canonical Wnt signaling by both translocating β-catenin to the nucleus and strongly upregulating Axin2 transcription (Fig. 6J-L). Inhibition of GSK3β by the chemical inhibitor BIO restores the nuclear localization of β-catenin protein in TKO MEFs (arrows). qPCR analysis of the canonical Wnt target gene Axin2 reveals the failure of rWNT3A to activate Axin2 transcription in TKO MEFs, which can be rescued by inhibition of GSK3β by BIO. qPCR analysis of chondrocyte (Sox9, Col2a1) and osteoblast (Runx2) lineage markers in wild-type versus TKO MEFs upon rWNT3A stimulation. Note the strong repression of chondrocytic fate in wild-type MEFs, which is mitigated by loss of LAR family phosphatase activity. Error bars indicate s.e.; *P<0.01, ***P<0.005.

**DISCUSSION**

Genetic inactivation of the genes encoding the LAR family receptor protein tyrosine phosphatases RPTP-σ and LAR revealed their functional redundancy in craniofacial development. Double-mutant embryos exhibit a shorter lower jaw (micrognathia), accompanied by a failure in tongue development and positioning.

**Fig. 6. Decrease in canonical Wnt/β-catenin signaling in cells lacking LAR family phosphatases.** (A,B) Immunofluorescent staining for active β-catenin protein in control (A) and Ptprs−/−;Ptprf−/− (B) transverse mandibular (Md) arch sections at E10.5. A strong downregulation of active β-catenin is observed throughout the mutant mandibular arch. (C,D) In situ hybridization for Axin2 expression in control (C) and Ptprs−/−;Ptprf−/− (D) mandibular arches. Sections are adjacent to A,B and confirm the loss of canonical Wnt signaling in the mutant. (E) qPCR analysis of disseected control and Ptprs−/−;Ptprf−/− mandibular arches at E10.5 reveals significant downregulation of the canonical Wnt target genes Axin2 and cyclin D1 (Cdcn1) in mutant arch tissue. (F-K) Immunofluorescent staining for β-catenin in wild-type (F,J) and Ptprs−/−;Ptprf−/−;Ptprd−/− TKO (G,I,K) MEFs. (F,G) β-catenin fails to translocate to the nucleus in mutant MEFs treated with recombinant (r) WNT3A (arrows, F,G). (J,K) Inhibition of GSK3β by the chemical inhibitor BIO restores the nuclear localization of β-catenin protein in TKO MEFs (arrows). (L) qPCR analysis of the canonical Wnt target gene Axin2 reveals the failure of rWNT3A to activate Axin2 transcription in TKO MEFs, which can be rescued by inhibition of GSK3β by BIO. qPCR analysis of chondrocyte (Sox9, Col2a1) and osteoblast (Runx2) lineage markers in wild-type versus TKO MEFs upon rWNT3A stimulation. Note the strong repression of chondrocytic fate in wild-type MEFs, which is mitigated by loss of LAR family phosphatase activity. Error bars indicate s.e.; *P<0.01, ***P<0.005.
(microglossia/glossoptosis) and cleft palate. These defects are caused by a combination of aberrant mandibular patterning and proliferative signaling cues provided by the Wnt and BMP pathways. Importantly, the phenotypic triad of micrognathia, glossoptosis and cleft palate observed in LAR family phosphatase-deficient mice at birth is reminiscent of human PRS.

We previously reported that 45% of DKO embryos harbor micrognathia at E18.5 (Uetani et al., 2009). The basis of this incomplete penetrance is unclear but our results show that the phenotype is already present at E10.5, which implies that the threshold effect on craniofacial morphogenesis occurs prior to this stage. Furthermore, in the present study we found that all Ptprs;Ptprf mutants with micrognathia also had microglossia/glossoptosis and cleft palate. Conversely, none of the Ptprs;Ptprf mutants without micrognathia had tongue or palate malformations. The strong association between these phenotypes points to a single developmental origin for all three malformations. Since we do not observe early PA1 defects, notably in regard to NCC migration or survival, and as cleft palate or glossoptosis is unlikely to cause micrognathia, the defect in mandibular development is most likely the primary cause of the PRS-like phenotype in DKO embryos. Accordingly, the patterning and proliferation defects occur earlier and are more pronounced in this compartment. The mandibular origin of the PRS triad was also postulated in humans and in previous mouse models (Ricks et al., 2002; Evans et al., 2011). However, as Ptprf is also expressed in the palatal epithelium and Ptprs is found at lower levels in this region, we cannot exclude the possibility of additional roles for these phosphatases in the maxillary compartment that would sensitize this tissue to palatal shelf defects.

The close resemblance between the LAR family-deficient phenotype and human PRS suggests that the signaling pathways and downstream targets regulated by LAR phosphatases might be involved in PRS etiology. Here, we identified signaling defects in the BMP and Wnt patterning pathways, which have previously been reported to affect mandibular and maxillary development. Of interest, the loss of Wnt pathway components, including LRP6, GSK3β, Rspo2 and β-catenin, results in a variable degree of mandibular hypoplasia and cleft palate linked to altered proliferation and apoptosis (Brault et al., 2001; Song et al., 2009; He et al., 2010; Jin et al., 2011; Sun et al., 2012). Importantly, the severity of jaw abnormality depends upon the timing and the tissue in which β-catenin signaling is blocked (Brault et al., 2001; Sun et al., 2012). Specific loss of β-catenin activity in PA1 results in the distal truncation of Meckel’s cartilage, whereas proximal structures remain unaltered, similar to the phenotypes described in Ptprs; Ptprf double-mutant embryos (Jin et al., 2011; Zhang et al., 2011; Sun et al., 2012). Furthermore, this phenotype has been linked to proliferation and differentiation defects. Hence, the regulation of Wnt signaling is likely to be an important aspect of LAR family phosphatase function in mandibular development. Interestingly, our results show that GSK3β inhibition is sufficient to rescue β-catenin activation and rescue Axin2 transcription to near wild-type levels upon Wnt stimulation. This suggests that LAR phosphatases regulate the β-catenin degradation complex rather than upstream signal transduction. Further studies will be required to elucidate the detailed mechanism of canonical Wnt/β-catenin regulation by LAR family phosphatases.

In addition to Wnt defects, Ptprs; Ptprf mutant mice show an increase in the BMP signaling response. It is intriguing that the aberrant differentiation of Meckel’s cartilage that takes place in the LAR family-deficient mandible occurs in the region of increased BMP signaling. BMP4 expression has indeed been demonstrated to upregulate both chondrogenic and osteogenic cell fates (Semba et al., 2000; Yoon et al., 2005; Merrill et al., 2008; Bonilla-Claudio et al., 2012; Kumar et al., 2012). In particular, inducible overexpression of BMP4 results in a shortening of the mandible and expansion of Meckel’s cartilage (Bonilla-Claudio et al., 2012). Hence, the increase in BMP signaling in Ptprs;Ptprf-deficient embryos is also in line with the observed mandibular phenotype.

Given the concomitant regulation of both the BMP and canonical Wnt signaling pathways by the LAR family phosphatases, one outstanding question concerns the potential for secondary phenotypes due to interaction between the pathways. During PA1 morphogenesis, BMP signaling is active mainly in the distal mandibular mesenchyme (Fig. 5) (Liu et al., 2005; Bonilla-Claudio et al., 2012), while canonical Wnt signaling is detected throughout the proximal-distal axis of the mandibular arch (Fig. 6) (Mani et al., 2010). Hence, based solely on their expression, it is unclear whether these pathways interact in mandibular arch development. However, the fact that simultaneous activation of both the BMP and Wnt pathways in LAR family-deficient fibroblasts was unable to restore SMAD1/5 phosphorylation to wild-type levels and, conversely, that β-catenin activation was unaffected by rBMP4 treatment of wild-type MEFs (data not shown), argues against a secondary phenotype due to pathway interactions. Hence, it is plausible that LAR family phosphatases affect the Wnt and BMP signaling pathways independently and that the combined effect of their misregulation leads to the Meckel’s cartilage patterning defects. Our results and those of others (Merrill et al., 2008; Bonilla-Claudio et al., 2012; Kumar et al., 2012) show that mandibular distal BMP promotes cartilage marker gene expression, whereas Wnt signaling represses these genes (Hill et al., 2005; Zhang et al., 2011). In LAR family-deficient cells, the chondrocyte markers Sox9 and Col2a1 were strongly activated by BMP signaling, while their repression by Wnt signaling was impaired. This suggests a model in which the combined effect of the misregulation of both pathways impairs cartilage formation in the mandible of Ptprs;Ptprf mutant embryos. As a result, bone formation, which requires Meckel’s cartilage (Ito et al., 2002), would occur abnormally (supplementary material Fig. S6). In accordance with a secondary effect on bone formation, loss of LAR family phosphatases had a limited effect on the osteoblast marker Runx2 in isolated cells. Notably, neither Ptprs nor Ptprf is expressed in Meckel’s cartilage or mandibular bone, supporting a patterning defect rather than a cell-autonomous cartilage or bone differentiation defect.

In addition to patterning defects, the decrease in proliferation observed in double-mutant embryos is likely to exacerbate the mandibular growth phenotype. Proliferation has indeed been identified as a driving force in mandibular elongation (Ito et al., 2002). Proliferation is affected throughout the mandibular region where Wnt/β-catenin signaling is impaired, which correlates with the loss of Ccnd1 expression, a cell cycle component regulated by Wnt signaling. As the LAR family members are expressed in PA1 mesenchyme at E9.5-10.5, they could directly affect Wnt signaling in these cells to impact proliferation. The later proliferation defects in these tissues might result from the loss of low-level LAR family expression in the more differentiated structures or from secondary mechanisms arising from earlier patterning deficiencies (supplementary material Fig. S6).

In summary, we propose that Ptprs;Ptprf double-mutant mice provide important insight into understanding the etiology of PRS, specifically by highlighting the crucial role of mandibular patterning and proliferation control by the Wnt and BMP signaling pathways.
Accordingly, mutations in BMP2 and in the COL2A1 regulator SOX9 have been identified in PRS (Snead and Yates, 1999; Benko et al., 2009; Sahoo et al., 2011). These results suggest the LAR family phosphatases as candidate PRS genes in humans and prompt a greater interrogation of the BMP and Wnt pathways in PRS patients.

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The authors declare no competing financial interests.

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K.S. conceived and performed experiments; W.H. provided critical materials; M.B. provided financial support; and K.S. conceived the experiments and wrote the manuscript.

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References

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Development

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LAR phosphatases in the mandible


