A role for suppressed incisor cuspal morphogenesis in the evolution of mammalian heterodont dentition

Atsushi Ohazama[a], James Blackburn[a], Thantrira Porntaveetus[a], Masato S. Ota[b], Hong Y. Choi[c], Eric B. Johnson[c], Philip Myers[d], Shelly Oommen[a], Kazuhiro Eto[b], John A. Kessler[e], Takashi Kondo[f], Gareth J. Fraser[g,1], J. Todd Streelman[g], Ulyses F. J. Pardiñas[h], Abigail S. Tucker[a], Pablo E. Ortiz[i], Cyril Charles[j], Laurent Viriot[k], Joachim Herz[c], and Paul T. Sharpe[a,2]

[a]Department of Craniofacial Development, Dental Institute, King's College London, Guy's Hospital, London SE1 9RT, United Kingdom; [b]Section of Molecular Craniofacial Embryology, Graduate School, Tokyo Medical and Dental University, Tokyo 113-8549, Japan; [c]Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX 75390-9046; [d]Department of Ecology and Evolutionary Biology, Museum of Zoology, University of Michigan, Ann Arbor, MI 48109-1079; [e]Department of Neurology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611; [f]Kondo Research Unit, Neuro-Developmental Disorder Research Group, Brain Science Institute, Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198, Japan; [g]School of Biology, Georgia Institute of Technology, Atlanta, GA 30332; [h]Centro Nacional Patagónico, 9120 Puerto Madryn, Chubut, Argentina; [i]Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina, Cátedra de Paleontología, Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán, Miguel Lillo 205 4000–San Miguel de Tucumán, Argentina; [j]Department of Orofacial Sciences, University of California, San Francisco, CA 94143-0442; and [k]Team «Evo-Devo of Vertebrate Dentition», Institute of Functional Genomics of Lyon, Université de Lyon, Centre National de la Recherche Scientifique, Institut National de la Recherche Agronomique, 69364 Lyon Cedex 07, France

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Changes in tooth shape have played a major role in vertebrate evolution with modification of dentition allowing an organism to adapt to new feeding strategies. The current view is that molar teeth evolved from simple conical teeth, similar to canines, by progressive addition of extra "cones" to form progressively complex multicuspid crowns. Mammalian incisors, however, are neither conical nor multicuspid, and their evolution is unclear. We show that hypomorphic mutation of a cell surface receptor, Lrp4, which modulates multiple signaling pathways, produces incisors with grooved enamel surfaces that exhibit the same molecular characteristics as the tips of molar cusps. Mice with a null mutation of *Lrp4* develop extra cusps on molars and have incisors that exhibit clear molar-like cusp and root morphologies. Molecular analysis identifies misregulation of Shh and Bmp signaling in the mutant incisors and suggests an uncoupling of the processes of tooth shape determination and morphogenesis. Incisors thus possess a developmentally suppressed, cuspid crown-like morphogenesis program similar to that in molars that is revealed by loss of Lrp4 activity. Several mammalian species naturally possess multicuspid incisors, suggesting that mammals have the capacity to form multicuspid teeth regardless of location in the oral jaw. Localized loss of enamel may thus have been an intermediary step in the evolution of cusps, both of which use *Lrp4*-mediated signaling.

cusp | Lrp4 | tooth development | evo/devo | multicuspid crown

Vertebrates exhibit remarkable diversity in their dentitions, which is a feature of the importance of tooth shape in adaptation to new feeding strategies in evolution. Even quite closely related species of mammals can have different shapes of teeth and thus tooth development provides an excellent model for molecularly based evolutionary developmental biological studies (evo/devo). These tooth evolutional changes took place by the activation or inactivation of gene function, and thus evolutionary lost structures or gene activation/inactivation during evolution are occasionally retained as vestigial structures or latent gene activation/inactivation at embryonic stages.

The current view is that all mammalian teeth evolved from simple ancestral teeth with a conical shape not dissimilar to mammalian canines (1). Mammalian heterodont dentitions contain a variety of tooth shapes and most evo/devo studies have focused solely on the molar dentition, with cuspal morphology being used as the main comparative feature between specimens (1). A cusp is a pointed or rounded projection of the tooth that is composed of both enamel and dentin, and the general consensus is that multicuspid teeth (molariform) evolved from conical teeth by progressive addition of extra "cones" (1). Incisors however are a uniquely mammalian tooth type that are neither conical nor multicuspid and their evolutional process is not understood.

Among mammalian teeth, murine dentition has been used as a powerful tool for evo/devo studies because of the relative ease of gene manipulation. A major defining feature of *Rodentia* is the presence of continuously growing incisors. Most mammalian teeth consist of a clearly recognizable crown that consists of a thin coating of enamel covering a thicker layer of dentine, and roots that are composed only of dentine that is often surrounded by an external layer of a supporting tissue (e.g., periodontal ligament). Rodent incisors, however, have no obvious crown or roots but have two distinct surfaces: a labial surface of enamel-covered dentine and lingual surface of dentine only. It has been suggested that the labial side corresponds to the crown and the lingual side corresponds to the root (2, 3).

The low-density lipoprotein (LDL) receptor family is a large, evolutionarily conserved group of transmembrane proteins (4, 5). The LDL receptor was first identified as an endocytic receptor that transports the lipoprotein LDL into cells by receptor-mediated endocytosis. More recent findings have shown that LDL receptor family members can also function as direct signal transducers or modulators for a broad range of cellular signaling pathways (6–9).

We show here that rodent incisors possess a developmentally suppressed, cuspid crown–like morphogenesis program that is revealed by loss of Lrp4 activity. *Lrp4* is thus responsible for maintaining the simple shape of incisors by suppression of cusp formation in development, a process that uncovers a likely route of mammalian incisor evolution.

Results and Discussion

The incisors of laboratory mice (*Mus musculus*) have smooth enamel surfaces (Fig. 1*A*). Mice with a hypomorphic mutation in

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[1]Present address: Department of Animal and Plant Sciences, Alfred Denny Building, University of Sheffield, Western Bank, Sheffield S10 2TN, United Kingdom.

[2]To whom correspondence should be addressed. E-mail: paul.sharpe@kcl.ac.uk.

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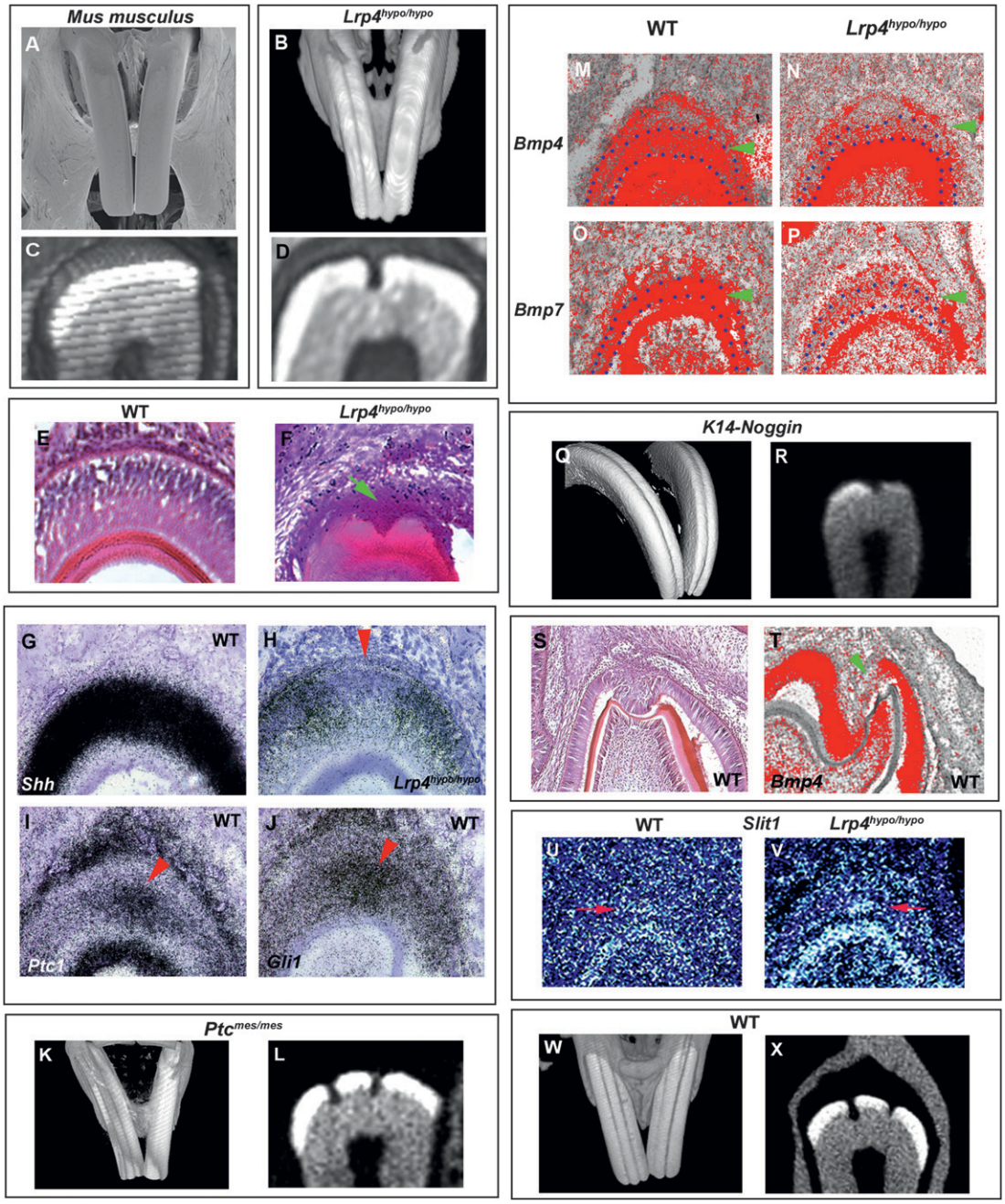

Fig. 1. Grooves in incisors of mutant mice. (*A* and *B*) Grooved incisors are found in *Lrp4*$^{hypo/hypo}$ (*B*), whereas there are no grooves in wild-type laboratory mice (*Mus musculus*; *A*). (*C* and *D*) Cross sections of incisors showed that grooves were caused by lack of enamel (*D*). (*E* and *F*) The first sign of grooves was at postnatal day 5 (P5) in *Lrp4*$^{hypo/hypo}$ mice (arrow in *F*). (*G–J*) *Shh* expression was downregulated at the presumptive groove region in *Lrp4*$^{hypo/hypo}$ mice at birth (arrowhead in *H*) whereas strong *Ptc1* and *Gli1* expression was observed in a similar region in wild-type (arrowhead in *I* and *J*). (*M–P*) Downregulation of *Bmp4* (arrowhead in *N*) and *Bmp7* (arrowhead in *P*) expression was observed in ameloblasts in *Lrp4*$^{hypo/hypo}$ mice at birth. (*K, L, Q,* and *R*) *Ptc*$^{mes/mes}$ (*K* and *L*) and *K14-Noggin* (*Q* and *R*) mice showed labial grooves that were caused by the lack of enamel. (*S*) Molar enamel–free zone in wild-type laboratory mice at P2. (*T*) Reduction in *Bmp4* expression was observed at enamel-free zones at P2 (arrowhead). (*U* and *V*) Enamel-free zone marker gene, *Slit1* was expressed at the presumptive groove region in *Lrp4*$^{hypo/hypo}$ mice at birth (arrow in *V*), whereas very faint *Slit1* expression could be detected in similar regions in wild-type (arrow in *U*). (*A–D, K, L, Q,* and *R*) Images of incisors obtained from 3-month-old animals. (*W* and *X*) Incisors of 2-year-old wild-type laboratory mouse. Three-dimensional reconstructions (*B, K, Q,* and *W*) and cross-section (*C, D, L, R,* and *X*) based on micro-CT scans and SEM images (*A*) of maxillary incisors. Developing upper incisors (*E–J, M–P, U,* and *V*) and lower molars (*S* and *T*). *Lrp4*$^{hypo/hypo}$ (*B, D, F, H, N, P,* and *V*) and wild-type mice (*A, C, E, G, I, J, M, O,* and *S–U*). Ameloblasts are outline in blue (*M–P*).

the LDL receptor 4 (*Lrp4*; also known as *Megf7*; *Lrp4*$^{hypo/hypo}$) showed distinct grooved incisor labial surfaces (Fig. 1*B*). Cross section analysis of the grooved incisors of *Lrp4*$^{hypo/hypo}$ mice showed that the grooves were caused by a reduction of enamel on the labial surface (Fig. 1*D*).

Examination of the development of labial grooves in the mutants showed these to first appear shortly after birth; therefore we searched for molecular changes at this stage that might reveal a possible mechanism for the loss of enamel (Fig. 1*F*). The Shh pathway plays a critical role in ameloblast differentiation, the

cells that co-ordinate enamel formation (10, 11). In frontal sections of wild-type laboratory mice, the *Shh* receptor *Ptc1* was expressed in ameloblasts at the presumptive groove region, and *Shh* was expressed uniformly in ameloblasts (Fig. 1 *G* and *I*). *Gli1* was also strongly expressed at the presumptive groove region (Fig. 1*J*). This indicates Shh activity in the ameloblasts of wild-type incisors at the position where the groove forms in the mutants. In the *Lrp4*$^{hypo/hypo}$ mutant, *Shh* expression in ameloblasts was generally downregulated, but a clear area of greatly reduced expression corresponded to the site of groove formation (Fig. 1*H*). An accompanying downregulation of *Ptc1* and *Gli1* expression was also observed at the presumptive groove region in *Lrp4*$^{hypo/hypo}$ mice (Fig. S1). To establish any causal link between loss of Shh activity and groove formation, we analyzed mice with mutations in the Shh pathway that survive after birth. The spontaneous mouse mutant, *mesenchymal dysplasia*, has an abnormal C terminus of the Ptc1 protein (*Ptc*$^{mes/mes}$) that changes Shh activity (12–14). The maxillary incisors of these mice had grooves on their labial surfaces that were also caused by a lack of enamel (Fig. 1 *K* and *L*). This suggests that lowering of Shh activity in postnatal ameloblasts can lead to localized loss of enamel, in turn leading to the formation of labial grooves.

BMP Signaling Has Been Shown to Induce Ameloblast Differentiation (15). We found *Bmp4* and *Bmp7* expression to be specifically downregulated in ameloblasts of *Lrp4*$^{hypo/hypo}$ mutants at birth whereas expression was unaltered in odontoblasts (Fig. 1 *N* and *P*). Significantly, mice overexpressing the BMP antagonist, *Noggin* under the keratin 14 promoter (K14-Noggin) also showed grooves on the labial surface of maxillary incisors that were caused by loss of enamel (Fig. 1 *Q* and *R*). Thus, changes in both Shh and Bmp activity can lead to localized loss of enamel and groove formation.

The lack of enamel at the incisor grooves is reminiscent of the enamel-free zones located at the tip of the cusps of rodent molars that result from failure of complete ameloblast maturation (16–18). Before eruption of rodent molar teeth, the enamel-free zones are covered by ameloblasts, similar to those observed in the grooved incisors of *Lrp4*$^{hypo/hypo}$ mice (Fig. 1 *F* and *S*). To determine whether a conserved mechanism exists between lack of enamel on molar cusp tips and on grooved incisors, we compared the expression of genes known to be expressed during ameloblast differentiation in molars. Downregulation of *Bmp4* expression, seen in the presumptive groove region of *Lrp4*$^{hypo/hypo}$ mice, was also observed in ameloblasts covering the enamel-free zones of wild-type molar teeth (Fig. 1*T*). A more specific marker of the enamel-free zone is expression of *Slit1,* which shows a localized patch of expression in ameloblasts at the tips of developing molar cusps (19, 20). Weak *Slit1* expression was observed in odontoblasts of wild-type mouse incisors with a very small faint patch of expression at the groove location site at birth (Fig. 1*U*). In *Lrp4*$^{hypo/hypo}$ mice, *Slit1* expression was increased in odontoblasts, and the patch of expression in ameloblasts at the groove location site was clearly visible (Fig. 1*V*). Thus the site of the formation of incisor labial grooves shares molecular characteristics with the molar enamel-free zone. Alteration of Shh or Bmp signaling pathways either directly (*Ptc*$^{mes/mes}$*, K14-Noggin*) or indirectly via hypomorphic mutation of *Lrp4* reveals the cryptic incisor enamel-free zone. The existence of a very weak patch of *Slit1* expression in wild-type incisors at the position where a groove forms in the mutants, together with expression of *Ptc1* and *Gli1* in this same small region, all of which show changes in *Lrp4*$^{hypo/hypo}$ mice, suggests that this region of ameloblasts may be different from all other ameloblasts in the incisors. The obvious interpretation of these expression patterns is that ameloblasts in this region have compromised mineralization capacity. However, no obvious changes in enamel across the width of wild-type incisors have ever been reported. Based on the very weak patch of Slit1 expression observed in wild-type incisors (Fig. 1*U*), we reasoned that enamel might be susceptible in this area. We also reasoned that any defect in the ability of these cells to form enamel would be small and thus might only be evident in older mice. We thus analyzed the incisors of wild-type C57/BL6 mice that were 2 years old, and found very clear evidence of labial grooves as a result of a lack of enamel (Fig. 1 *W* and *X*). This suggests that the ameloblasts that coordinate enamel formation in the region of the groove become defective with age. Because ameloblasts in murine incisors are continually produced from the cervical loop stem cells, this may indicate age-related and location-specific defects in these cells (21).

Lrp4$^{hypo/hypo}$ mice often show supernumerary maxillary incisors (37%) whereas almost all of *Lrp4* null mice exhibit supernumerary maxillary incisor tooth germs at birth (Fig. S2) (8). It has been shown that the extra incisor tooth germs grow from endogenous incisor tooth germs (22). However, grooved incisors were found in newborn *Lrp4*$^{hypo/hypo}$ mice that did not have the supernumerary maxillary incisors and were also found in wild-type old mice (Fig. S2). This excludes the possibility that the groove is formed by a failure of the separation of supernumerary incisor tooth germs from endogenous tooth germs.

Several rodent species have been found to have grooved incisor labial surfaces. Species such as the meadow jumping mouse (*Zapus hudsonius*) and the cane rat (*Thryonomys swinderianus*) have one (Fig. 2*A*) to three (Fig. 2*B*) vertical grooves on the labial surfaces of their maxillary incisors, whereas other species such as grooved-toothed rats (*Otomys tropicalis*) display labial grooves in both maxillary and mandibular incisors (Tables S1–S3). Although rodents mostly have smooth incisors, we found grooved incisors in 60 rodents among 300 species investigated (Fig. S3 and Tables S1–S3). Labial grooves are also seen in species usually considered to be outside what are strictly considered as Rodentia, namely lagomorphs (picas, rabbits, and hares), which also possess lifelong continuously growing incisors (Fig. 2*C*). Cross-section analysis of the grooved incisors of several wild-type rodent species showed that the grooves were caused by a reduction of enamel on the labial surface (Fig. 2 *D*–*F*). Some fossil rodents also had grooved incisors, indicating that the labial grooves may have been lost in certain rodents, including *Mus musculus*, during evolution (Figs. S3 and S4) (23). In addition to rodent teeth, the enamel-free zone can also be observed in African cichlid fishes, suggesting that enamel-free zones are conserved structures in vertebrates (Fig. 2 *G* and *H*).

Some naturally occurring incisor grooves in several rodent species were, however, not found to be caused by loss of enamel but rather by folding of the enamel/dentin, similar to those observed in molar cusps (Fig. 3). When we examined the incisors of *Lrp4* null mutant mice, we found a more severe phenotype than *Lrp4*$^{hypo/hypo}$ mice. *Lrp4* null mutant incisors exhibited folded enamel and dentin on the labial side that was not observed in *Lrp4*$^{hypo/hypo}$ mice (Fig. 4*B*). *Slit1*, a marker of tertiary enamel knots as well as the enamel-free zone, was found in the tooth epithelium corresponding to the folded enamel/dentin in the mutant incisors (Fig. 4*C*) (19, 20). This suggests that the folded enamel/dentin represents a cusp-like structure. These mutant incisors are thus reminiscent of multicuspid crowns. Interestingly, multiple grooves (enamel-free zones) were also occasionally observed in *Lrp4*$^{hypo/hypo}$ and several rodent species (Fig. 2 *B* and *I*). In molars, cusp formation is initiated by a transient epithelial structure, the primary enamel knot (24, 25). To establish any link between *Lrp4*, primary enamel knots and cusp morphogenesis, we examined the expression of *Lrp4*, *Wnt10b,* and *p21* during incisor development. The enamel knot marker genes, *Wnt10b* and *p21* showed restricted coexpression with *Lrp4* in incisor tooth epithelium (Fig. 4 *D*–*F*). Interestingly, *Lrp4* expression was also observed in the primary enamel knot in the molars (8). These results suggest that the role of *Lrp4* in incisors

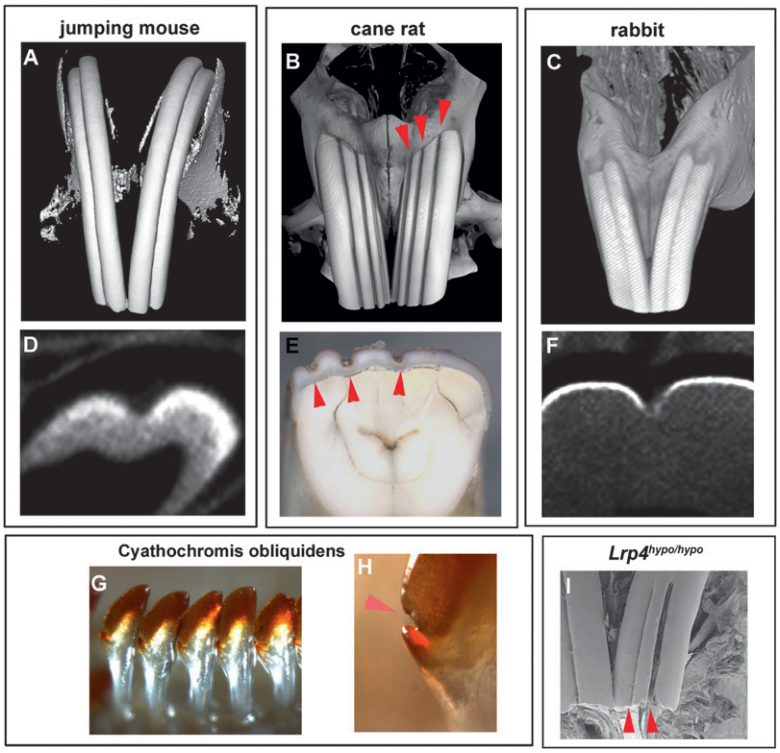

Fig. 2. Grooved incisors in various wild-type rodents, rabbits, and fish. (*A–C*) Grooved incisors are found in jumping mice (*Zapus hudsonius*; *A*), cane rat (*Thryonomys swinderianus*; *B*), and rabbit (*Oryctolagus cuniculus*; *C*). (*D–F*) Cross-sections of incisors showed that grooves were caused by lack of enamel. Multiple grooves were found in cane rat and in *Lrp4*$^{hypo/hypo}$ mice (arrowheads in *B, E,* and *I*). Three-dimensional reconstructions (*A–C*) and cross-section (*D* and *F*) based on micro-CT scans, SEM images (*I*), and stereomicroscopic images (*E*) of maxillary incisors. (*G* and *H*) Enamel-free zone in cichlid fishes (*Cyathochromis obliquidens*; arrowhead in *H*). (*A–F* and *I*) All images of incisors were obtained from 3-month-old animals.

is probably similar to that in molars. Interestingly, *Lrp4* null mice also had extra molar cusps, suggesting that *Lrp4* might have a general role in suppressing cusp formation (Fig. 4 *H, J,* and *L*).

In addition to the labial side showing multiple cusp-like structures, the lingual surfaces of the *Lrp4* null incisors were also very uneven, with protrusions producing a "corrugated" appearance (Fig. 4*B*). The lingual portion of the incisors also showed a discontinuity of epithelium that is not a normal feature of lingual incisor epithelium (Fig. 4 *M* and *N*). The apical edge of lingual epithelium histologically resembled an epithelial root sheath that is a unique structure found in developing molar tooth roots (Hertwig's epithelial root sheath; 3, 26). *Ptc2* expression, a marker of Hertwig's epithelial root sheath in molars (14), was clearly identified in the apical edge of epithelium of the incisors of *Lrp4* null mutants (Fig. 4*O*), suggesting that in the absence of *Lrp4*, epithelial cells on the lingual aspect of developing incisors were organized into root-forming structures usually seen in only molars. The existence of both a crown with multiple cusp-like structure and molar-type roots indicates that the *Lrp4* null mutant incisors have undergone a transformation toward molars. To investigate this further, we examined the expression of molar mesenchyme marker *Barx1* in the mutant incisors (27). *Barx1* expression could not be detected in the incisors of *Lrp4* null mice, suggesting that the mesenchyme of *Lrp4* null mutant incisors has retained its incisor identity (Fig. 4 *P* and *Q*). The existence of cusp-like structures with enamel-free zones in the epithelium of *Lrp4* null mutant incisors, together with the retention of incisor identity in the mesenchyme identifies an uncoupling of the processes of tooth shape determination (mesenchyme) and morphogenesis (epithelium). Interestingly, multicuspid incisors that are composed of folded enamel/dentin with enamel-free zones are naturally found in some mammalian species (Fig. 4 *R–U*). Furthermore, several extinct mammalian species also had multicuspid incisors (28–33).

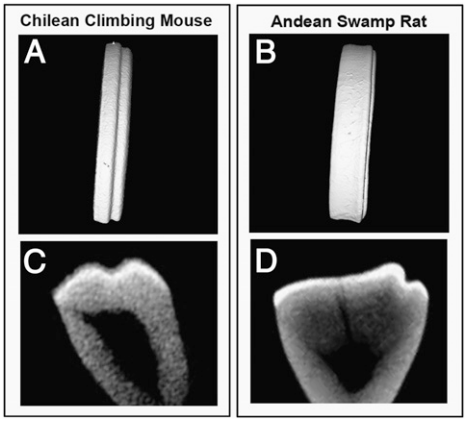

Fig. 3. Folding enamel/dentin in rodent incisors. Incisors with grooves caused by folded enamel/dentin in wild-type rodent species (Chilean climbing mouse; *A* and *C*) and Andean swamp rat; *B* and *D*). Three-dimensional reconstructions (*A* and *B*) and cross-section (*C* and *D*) based on micro-CT scans of maxillary incisors.

The evolutional processes of deriving complex heterodont mammalian dentitions from simple conical-shaped teeth has been much discussed in the last century (1, 34). Gaining cusps has been established as a major event in mammalian evolution. In East African cichlid fish, some species show tooth crown shape reversal where monocuspid teeth evolved from multicuspid teeth (35, 36). Interestingly, dolphins also possess a homodont conical tooth dentition whereas the primitive eutherian heterodont dentition included multicuspid teeth (1). *Lrp4*$^{hypo/hypo}$ mice show enamel-free zones in in-

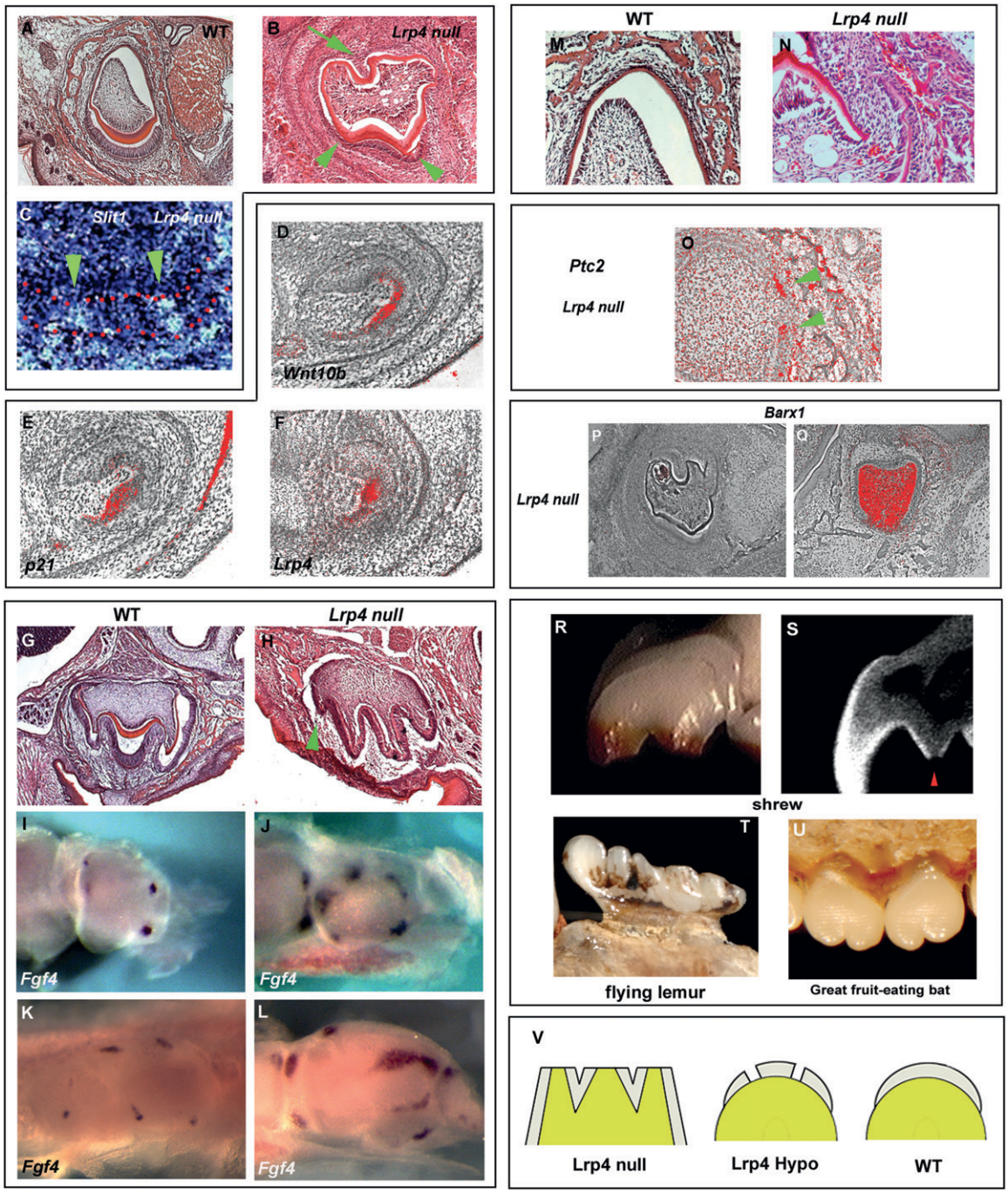

Fig. 4. Cusp-like structure and molar-type roots in mammalian incisors. (*A* and *B*) *Lrp4 null* mutants showed folding of enamel/dentin on the labial sides of incisors at P1 (arrowheads in *B*). Molar root-like structures were also found at the lingual side of incisors in *Lrp4 null* mutants (arrow in *B*). (*C*) *Slit1* a marker of tertiary enamel knot and the enamel-free zones, was found in the tooth epithelium corresponding to the folded enamel/dentin (arrowheads in *C*). Ameloblasts are outlined in red (*C*). (*D–F*) Expression of enamel knot marker genes (*Wnt10b*; *D*) and (*p21*; *E*), and *Lrp4* expression (*F*) in lower incisors at E13.5 in wild type. (*G* and *H*) Extra cusps in upper molar of *Lrp4* null mutant (arrowhead in *H*). (*I–L*) Extra *Fgf4* expression domain was found in second lower molar (*J*) and first lower molars (*L*) of *Lrp4* null mice. (*M* and *N*) Some incisors showed a gap at the lingual side in *Lrp4* null mutants (*N*). (*O*) *Ptc2* expression was observed at the gap (arrowheads). (*P* and *Q*) *Barx1* expression in lower incisor (*P*) and lower molar (*Q*) of *Lrp4* null mutant at P1. (*R–U*) Multicuspid incisors of shrews (*Sorex arcticus*; *R* and *S*), flying lemurs (*Galeopterus variegates*; *T*), and great fruit-eating bats (*Artibeus lituratus*; *U*). (*S*) Cross-section based on micro-CT scans of maxillary incisors, showing folded enamel/dentin with enamel-free zone (arrowhead). (*V*) Diagram of evolutionary mutant/modification series; incisor of Lrp4 null mutant (*Left*), *Lrp4*$^{hypo/hypo}$ (*Center*), and wild-type (*Right*) mice. (*A–H* and *M–Q*) Frontal sections. Wild-type mice (*A, D–G, I, K,* and *M*) and *Lrp4* null mutant (*B, C, H, J, L,* and *N–Q*).

cisors and *Lrp4* null mice exhibit a more severe phenotype of incisors with multiple cusp-like structures with enamel-free zones (Fig. 4*V*).

Lrp4 mutations reveal a developmentally suppressed program of molar-like epithelial changes in incisors. In some species this program is less suppressed, leading to incisors with obvious cuspid-like morphologies. Naturally occurring grooved incisors may represent a vestigial remnant of this suppressed molar program. Multiple signaling pathways are involved in cusp formation; in this

context it is significant that *Lrp4* is a known direct mediator of both Wnt and Bmp signaling and an indirect mediator of Shh (8, 9) and thus may have played a pivotal role in evolution of heterodontia (Fig. 4*V*). Lrp4 via its action on multiple signaling pathways including Shh, Bmp, and Wnt is thus central to a transition between a continuous enamel covering, grooved enamel, and folded enamel, all of which appear in the mammalian fossil record and extant species and raise the question of whether enamel grooves proceeded enamel folds in the evolution of multicuspid teeth.

Materials and Methods

Production and Analysis of Mutant Mice. *Lrp4*$^{hypo/hypo}$ mice were produced as described by Johnson et al. (9). *Lrp4* null mice were generated by deletion of the transcription start site and exon 1, which encodes the signal peptide and the initiating ATG. This strategy ensures that no residual functional protein can be generated. *Ptc*$^{mes/mes}$ were produced as described by Makino et al. (13). *K14-Noggin* were produced as described by Guha et al. (37).

In Situ Hybridization. Whole-mount and radioactive section in situ hybridization was carried out using DIG labeled or ^{35}S-UTP radiolabeled riboprobes (8) that were generated from mouse cDNA clones that were gifts from several laboratories: *Fgf4* (G. R. Martin, University of California San Francisco), *Ptc2* (A. Gritli-Linde, Göteborg University), and *Shh* (A. P. McMahon, Harvard University).

Scanning Electron Microscope Analysis. Jaws were coated with gold and photographed using standard scanning electron microscopy.

Micro CT Analysis. Heads of mice were scanned with Explore Locus SP (GE Preclinical Imaging) high-resolution micro-CT with a voxel dimension of 8 μm. Three-dimension reconstruction was performed by three structure analysis software, Microview (GE Preclinical Imaging).

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