hypothesis because it indicates that the parental magma is not particularly evolved.

There are two observations that are inconsistent with the hypothesis that KREEP initiates melting of mafic cumulates to produce young lunar magmas. First, there are no samples with ancient ages and large KREEP geochemical signatures in the sample collections. However, this could reflect a sampling bias introduced by the fact that all ancient KREEP-rich igneous samples are derived from two closely related plutonic suites[22]. The second observation is that radiometric ages determined on low- and high-Ti basalts that lack the KREEP geochemical signature are often relatively young (3.1 to 3.4 Gyr; refs 16, 24). This implies that another mechanism to melt sources with small amounts of KREEP-rich material is required. We speculate that KREEP-rich material is the heat source for these magmas as well. However, melting is not initiated at the site where this material resides; instead, heating of mafic cumulates by KREEP-rich materials promotes upwelling of diapirs in the mantle. Thus, melting could be initiated as pressure is released. If this mechanism is valid, it implies that KREEP-rich materials are directly or indirectly responsible for melting of the lunar mantle. □

Methods

The sample was washed and sonicated in four-times quartz-distilled water, followed by 0.5 M acetic acid. The sample was next crushed using a sapphire mortar and pestle, and sieved at 100–200 and 200–325 mesh. Mineral separations were begun using heavy liquids on both size fractions. Plagioclase floated in 2.85 g cm^{-3}, whereas the mafic minerals sank. Hand-picking was used to separate pyroxene (brown) and olivine (green) mineral grains, as well as to purify the plagioclase fraction. Individual mineral fractions were then leached in 1 N HCl for 10 min in a sonicator before digestion. Chemical separations and isotope ratio measurements were done at the Radiogenic Isotope Laboratory, University of New Mexico, following standard silicate dissolution procedures, and involved cation chromatography using a combination of HCl and methalactic acids. Isotopic ratios were measured on a Micromass Sector 54 multi-collector thermal ionization mass spectrometer on Faraday cups in static mode. Rubidium, Sr, Sm, and Nd blanks measured during the course of the investigation averaged 7, 12, 7 and 8 pg, respectively. Normalization and standard values are given in Table 1.

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Nonindependence of mammalian dental characters

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Studies of mammalian evolution frequently use data derived from the dentition[1–4]. Dental characters are particularly central for inferring phylogenetic relationships of fossil taxa[1–4], of which teeth are often the only recovered part. The use of different aspects of dental morphology as phylogenetic signals implies the independence of dental characters from each other. Here we report, however, that, at least developmentally, most dental characters may be nonindependent. We investigated how three different levels of the cell signalling protein ectodysplasin (Eda)[5] changed dental characters in mouse. We found that with increasing expression levels of this one gene, the number of cusps increases, cusp shapes and positions change, longitudinal crests form, and number of teeth increases. The consistent modification of characters related to lateral placement of cusps can be traced to a small difference in the formation of an early signalling centre at the onset of tooth crown formation. Our results suggest that most aspects of tooth shape have the developmental potential for correlated changes during evolution which may, if not taken into account, obscure phylogenetic history.

Although both new fossil and molecular data can be expected to resolve many of the phylogenetic incongruences[6–10], there is a continuing debate about the best way to use dental evidence in evolutionary taxonomy[6,9,10]. The 'total evidence' view assumes that covarying characters manifest phylogenetic congruence, but other views assume that covariance of characters can be misinterpreted as a phylogenetic signal for functional or developmental reasons. As part of the same individual ontogeny, all characters obviously have the potential for nonindependent, or correlated, changes during evolution, but whether this could be the case for most mammalian dental characters used in evolutionary taxonomy remains unresolved. Features of the mammalian dentition, as part

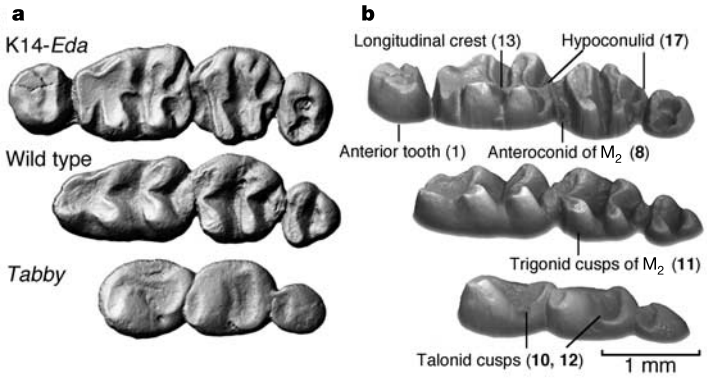
of a repetitively structured system, have classically been suggested to share the same quantitative genetic underpinnings[11–14]. Although an increasing number of heritable quantitative traits have been reported in teeth[15], most identified gene mutations with dental effects are characterized by a total or partial lack of teeth and are thus uninformative for the character analysis of tooth morphology. Here we quantified effects of a relatively mild genetic change on tooth shape to test whether and how dental characters used in evolutionary taxonomy might be developmentally linked.

We analysed how tooth characters and their development were altered in two mutant mouse strains compared to the teeth of normal 'wild type' mice (*Mus musculus*). One was a natural mutant, *Tabby*, which lacks functional ectodysplasin, and the other was a transgenic mouse, K14-*Eda*, which has superfluous expression of *ectodysplasin* under keratin-14 promoter in the basal epithelial cell layer[16]. We considered the *ectodysplasin* expression levels of the three mouse strains as character states of one developmental variable, and examined how the morphology of teeth change when ectodysplasin activity levels rise from zero (*Tabby*) to normal (wild-type controls), and beyond normal (K14-*Eda*).

Lack of functional ectodysplasin in humans causes an X-linked hypohidrotic ectodermal dysplasia syndrome (X-HED) in which the development of ectodermal organs like teeth, hair and skin glands is defective[17]. The mouse equivalent[5], *Tabby* mutant, was discovered more than 50 years ago and *Tabby* tooth morphology is characterized by missing cusps[17]. The presence of both human and mouse tooth phenotypes with reduced numbers of cusps and teeth due to *ectodysplasin* mutation, although not conclusive, is suggestive of the generality of the role of ectodysplasin in mammalian tooth development. Ectodysplasin, which is a member of the tumour necrosis factor (TNF) family of proteins, is a membrane protein which is cleaved to become freely diffusible in the extracellular space[17], and has been implicated in modulating growth and differentiation of most epithelial organs[17,18]. When the first morphologically distinguishable markers of developing teeth, dental placodes, appear, *ectodysplasin* expression is restricted to ectoderm that is adjacent to the tissues giving rise to teeth[18]. Ectodysplasin binds to its receptor Edar, which is expressed in dental placodes at the initiation of tooth development and in the enamel knots during crown formation[17,18]. The enamel knots, which express several signalling molecules, participate in regulating the formation of the tooth crown base and the individual cusps. The spatial patterns of enamel knots predict the species-specific cusp patterns[19].

We used conventional light and high-resolution three-dimensional laser confocal microscopy[20,21] to perform a character analysis. The results show that with increasing ectodysplasin signalling, many dental character states change beyond what is typically found within a species or even a genus (Fig. 1a). There is an overall increase in bucco-lingual distance between cusps. In *Tabby* the tips of the cusp pairs are typically fused, whereas in the wild-type mice the cusps are separated by an anteriorly oblique crest (Fig. 1a, b). In the K14-*Eda* mice, bucco-lingual separation of cusps has increased further with a straight transverse crest. Furthermore, in many teeth these transverse crests are connected by a longitudinal crest running along the midline of the crown (Fig. 1a, b). Longitudinal crests, not previously reported from mice or mouse mutants, are present in many other muroids and also in the Miocene members of the lineage leading to modern *Mus*[22]. The combination of straight lamellar-like transverse and central longitudinal crests is also typical of diprotodont marsupials (for example, kangaroos) and proboscideans (for example, African elephant). We note that first molars with a well-developed longitudinal crest connecting the protolophid to the hypolophid also have greater bucco-lingual cusp distances (Mann–Whitney U test, $P = 0.025$ to 0.035), suggesting that ectodysplasin signalling can cause the longitudinal crest formation indirectly, by affecting lateral placement of cusps.

A second distinct change correlating with the amount of ectodysplasin signalling is the number and shape of cusps. Compared to the anterior, trigonid part of the crown, the distal, talonid part is shallow in *Tabby* teeth (Fig. 1), and the distalmost cusp, the hypoconulid, is missing, whereas in the K14-*Eda* molars it has been incorporated into the wide posterior cingulid (Fig. 1). In addition to the missing hypoconulid, the characteristic anteroconid of the murid first lower molar is almost always missing in the *Tabby* mice. While these reductions in tooth crown features appear extreme, murids such as the water rats (genus *Hydromys*), with a



c

	Characters																		
"Taxon"	1	2	3	4	5	6	7	**8**	9	**10**	**11**	**12**	13	14	15	16	**17**	18	19
K14-*Eda*	(01)	1	3	1	2	(12)	(123)	3	(12)	2	2	2	(01)	0	2	2	2	(01)	(01)
Wild type	0	1	3	0	2	1	(12)	(01)	1	1	1	1	0	0	0	0	1	0	0
Tabby	0	(01)	(234)	0	0	(01)	(01)	0	(01)	0	(01)	0	0	0	0	0	0	0	0

Figure 1 Mouse molar teeth differ in several characters in mice with no (*Tabby*), normal (wild type), and above normal (K14-*Eda*) *ectodysplasin* activity. Occlusal (**a**) and obliquely lingual (**b**) views show right lower molar rows. Buccolingual cusp pairs of *Tabby* are fused, while these form a transverse loph in K14-*Eda*. Several other character states differ among teeth (**c**), such as the presence of a tooth anterior to first molar (character 1) and presence and shape of the hypoconulid (character 17). Many of the characters are polymorphic in both *Tabby* and K14-*Eda* mice (in parentheses). The non-polymorphic characters 10, 12 and 17, and also 8 and 11 with low degrees of polymorphism, are altered most consistently, owing to ectodysplasin signalling (shown in bold in **b** and **c**). For details, see Supplementary Information. M_2, lower molar.

primary diet consisting of fish and aquatic invertebrates[23], have molar morphologies reminiscent of *Tabby* molars. In contrast, the anteroconid of K14-*Eda* first molars have additional or better differentiated cusps. Limiting the size of the anteroconid anteriorly is an extra tooth in the K14-*Eda* mice in 58% of the tooth rows. This extra tooth is round and has one or two closely placed cusps. Its simple shape is reminiscent of the premolars of other rodent groups as well as premolars of the rodents implicated in the ancestry of muroids[22,24].

Our character state analysis shows that most of the *Tabby* and K14-*Eda* tooth features are polymorphic (Fig. 1c), indicating how an extreme drop or an increase in a gene activity may destabilize the developmental system. There is a tendency for more 'derived' character states to be present in the same tooth row but, in general, different parts of the crown have different sensitivities to changes in ectodysplasin signalling. Character states of the talonid were the least polymorphic, and the first molar anteroconid and the third molar were the most polymorphic (Fig. 1c). These differences in character polymorphism, here resulting from a complete gene deactivation or multiplication, suggest that evolutionary changes stemming from small tinkering of gene expression level may produce, at least initially, stepwise changes in only one or a few characters. Whereas different molecules are likely to affect character covariance differently, ectodysplasin modifies characters 8, 10, 11, 12 and 17 most consistently (Fig. 1b, c). All these characters appear to measure how well individual cusp tips are differentiated and separated bucco-lingually, suggesting a role for ectodysplasin in the regulation of the iterative process of cusp formation along the tooth row[19]. We hypothesize that these characters would be the first visible effects of small changes in ectodysplasin levels.

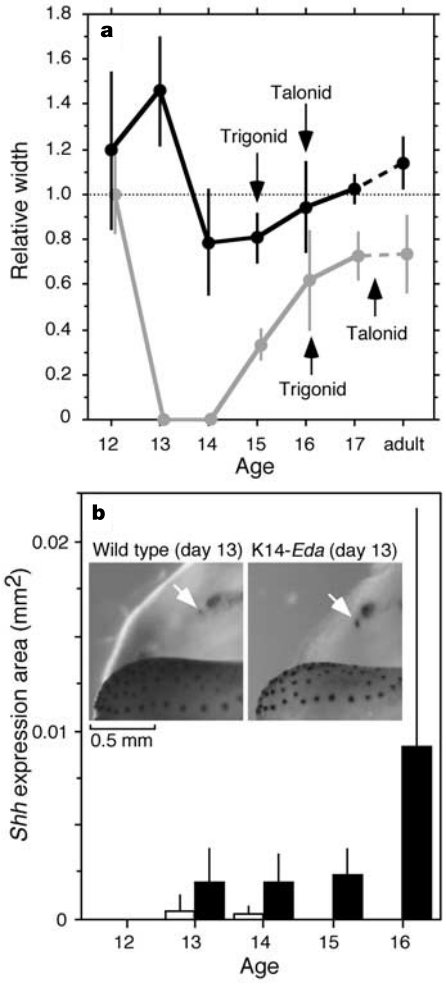


Figure 2 Dynamics of *Shh* expression in developing first molar (**a**) and extra tooth (**b**). Ratio-diagram of the width of *Shh* expression (cusps in adults) relative to wild-type molars (**a**) shows how the K14-*Eda* tooth expression domains (black line; error bars denote standard deviations) widen faster and surpass the wild type after day 17, while the *Tabby* tooth *Shh* expression domains (grey line) never reach the wild-type widths. *Shh* expression is not detected in *Tabby* from day 13 to 14 and in 40% of the wild-type teeth at day 13. *Shh* expression is continuous in K14-*Eda* teeth and relatively widest at day 12 and 13. All differences to the wild-type teeth are significant ($P < 0.05$, two-tailed test using 1,000 randomizations) except for *Tabby* at day 12 and K14-*Eda* at days 15 to 17. **b**, *Shh* is also upregulated (white arrows in the insets) anterior to the first enamel knot, transiently in wild-type mice (white bars) and prominently in the extra tooth of the K14-*Eda* mice (black bars). The developing taste papillae of the tongue express *Shh* (bottom of the insets).

Because our analyses show that several morphologically distinct characters can be altered as a function of changing expression level of a single gene, we next examined how the morphologies start to differ during development. We quantified the patterns of sonic hedgehog (*Shh*) expression from the onset of the first lower molar development. Shh is a signalling molecule required for the development of several organs and a marker for the overall signalling activity and size of the enamel knots[19]. In the enamel knots *Shh* expression allows the detection of lateral placement of the tooth cusps.

The results show that the earliest distinct differences in *Shh* expression domains are seen just before the formation of the first enamel knot (Fig. 2a). When the initial epithelial budding and first enamel knot appeared in wild-type mice, no *Shh* expression was detected in any of the studied *Tabby* tooth germs for two days. After this, *Shh* expression domains remain narrower relative to the wild-type molars throughout development and correspond in width to the fused cusps in fully formed teeth (Fig. 2a). In addition, the initiation of the talonid cusps of *Tabby* is delayed relative to the trigonid, resulting in the reduced talonid, analogous to the more primitive tribosphenic molar pattern. In contrast, in all K14-*Eda* mice studied, *Shh* expression continues uninterruptedly from the placode to the first enamel knot stage with initially a broader expression domain compared to the wild-type molars (Fig. 2a). The cusp-specific K14-*Eda Shh* expression of the following stage slightly lags behind that of the wild-type teeth, but widens faster as the teeth grow bigger (Fig. 2a). This delay in the widening of the K14-*Eda* first molar cusp spacing is concurrent with, and probably caused by, the expansion of the developing extra tooth anterior to it (Fig. 2b). These results indicate that modification of the forming primary enamel knot, from which the crown features develop, is enough to alter the states of several characters during the iterative process of cusp formation[19]. We interpret this to conform to the central role of morphodynamic[25] interplay between molecular signalling and growth, giving rise to the tooth shape. Thus, as shown here and supported by mathematical models[25], individual developmental variables can affect multiple characters simultaneously and each dental character does not necessarily require an individual 'genetic' code[11–15,25].

Although only about half of the K14-*Eda* tooth rows have an extra tooth, its development was initiated in all of the studied K14-*Eda* mice. However, the size of the *Shh* expression domain in the extra tooth is highly variable, suggesting that only tooth germs with the greatest initial knot activity develop into fully erupted extra teeth (Fig. 2b). This is indirectly supported by our discovery that wild-type mice have weak *Shh* expression activity in the same location at which the K14-*Eda* extra tooth develops (Fig. 2b). We note that although muroids have not developed lower premolars for over 45 million years[22,24,26] it is intriguing that the wild-type mice have transient upregulation of *Shh* in the ancient premolar location. The incipient and continuing development of the extra tooth in wild-type mice and the K14-*Eda* mice, respectively, does not conflict

with, and may support, the previous classification of minor epithelial invaginations anterior to the first molar as vestigial teeth[27]. In wild ground squirrels (genus *Spermophilus*), Goodwin showed supernumerary teeth to be more prevalent in hybrids between two species[28], suggesting a finely tuned role for genetic background in normal suppression of extra tooth development.

In conclusion, our analyses show correlated changes in dental characters as a function of quantitative changes in intercellular signalling. In the cases where the signalling activity of ectodysplasin, or of another equivalent molecule, can be modulated exclusively in the teeth, through tooth-specific *cis*-regulation, for example, these results suggest that most aspects of tooth shape have the potential for correlated changes during evolution. Our analyses clearly do not exclude the potential for dental characters to change independently in evolution. On the contrary, the disparate sensitivities of different dental characters to changes in ectodysplasin signalling suggest that gradual changes in dental morphology are possible even due to changes in the activity of a single gene. However, particularly when intermediate fossil morphologies are scarce, developmental non-independence should not be excluded from the hypotheses considered in evolutionary taxonomy. Sample sizes allowing, alternative character-coding strategies can be recommended, particularly when characters related to lateral cusp placement are found to covary. Such a case is the simplified tooth morphology of *Tabby* mice which suggests that extreme reduction of dental features in mammalian lineages such as seals and whales may have required only a simple developmental change. This would not only have rendered some of the characters interdependent, but also probably increased the likelihood of convergent evolution. From the palaeoecological point of view, developmental linkage of dental characters may have both facilitated, and directed, a relatively rapid increase in number of cusps and crests during periods of substantial environmental change. □

Methods

Mouse strains and morphological analysis

A transgenic mouse line has been recently created which expresses *ectodysplasin* (splice form A1) under keratin-14 promoter (K14)[16] in FVB/N mice (Jackson Laboratories). The *Tabby* allele was B6CBACa-A$^{w\text{-}J}$/A-Ta (stock no. JR 0314, Jackson Laboratories) and bred as described previously[18]. Wild-type mice were FVB/N and NMRI mice, which share similar character states. We analysed mice that lack or overexpress ectodysplasin (*Eda*) in preference to mice that lack or overexpress ectodysplasin receptor (*Edar*). There is no ectodysplasin signalling in either *Edar* null-mutants (*downless*) or *Tabby* mice (the *eda* null-mutant) and their teeth are morphologically similar. However, overexpressing ectodysplasin receptor under the K14 promoter will also change the pattern of cells responsive to ectodysplasin signalling, whereas the expression of *Edar* remains limited to enamel knots in the K14-*Eda* teeth. Thus, while K14-*Eda* mice can be considered as a character state change in only one developmental variable (signalling amount), K14-*Edar* mice represent character state changes both in strength of signalling and in distribution of responding cells. We note that K14-*Edar* tooth morphology is altered[29,30], but their morphology and pattern of variation differ from that of K14-*Eda* mice[30].

Here we analysed lower molars whose development is best understood. While upper molar morphology is also affected by ectodysplasin, only two of the K14-*Eda* mouse maxillae had an extra tooth. Thirty-six K14-*Eda*, 36 *Tabby*, and 34 wild-type (14 CBAT6T6 × NMRI and 20 FVB/N) tooth rows (both sides) were studied under a stereo microscope and photographed. Selected specimens were scanned using a laser confocal microscope at 8 μm *xyz*-resolution as described previously[20,21] except that the teeth were first coated with eosin in thin paraloid solution (Rohm and Haas). Three-dimensional models (DEMs) were made using a three-dimensional version of the National Institutes of Health IMAGE (http://www.physics.usyd.edu.au/physopt/3dview/).

Tooth characters and character states

The states of 19 dental characters were evaluated for the three strains, largely following Meng *et al.*[24], with some additional characters and character states included to cover the range of variation. We note that the characters are not meant as an exhaustive analysis of the dental features. Rather, they are used as representative characters that would be readily coded for fossil taxa (see Supplementary Information).

Developmental analysis

Whole-mount *Shh in situ* hybridization was performed using InsituPro robot (Intavis), using the protocol described earlier[16]. The stained mandibles and teeth were photographed and *Shh*-expressing areas in the developing teeth were measured. The number of specimens measured for day 12, 13, 14, 15, 16 and 17 *Tabby* mice were 16, 16, 28, 13, 33 and 10, respectively. The corresponding number of specimens for K14-*Eda* mice were 29, 29, 23, 8, 25 and 8, and for wild-type teeth 30, 20, 15, 32, 12 and 12. We used FVB/N (K14-*Eda* controls) as the wild-type controls owing to the smaller differences between the wild-type teeth and K14-*Eda* teeth. We randomized the mouse strain assignments 1,000 times to test differences in mean expression domains.

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