RSK2 Is a Modulator of Craniofacial Development

Virginie Laugel-Haushalter, Marie Paschaki, Pauline Marangoni, Coralie Pilgram, Arnaud Langer, Thibaut Kuntz, Julie Demassue, Supawich Morkmued, Philippe Choquet, André Constantinesco, Fabien Bornert, Matthieu Schmittbuhl, Solange Pannetier, Laurent Viriot, André Hanauer, Pascal Dolle, Agnès Bloch-Zupan

1 Institute of Genetics and Molecular and Cellular Biology (IGBMC), Centre National de la Recherche Scientifique (UMR 7104), Institut National de la Santé et de la Recherche Médicale (INSERM) U1121, University of Strasbourg, Illkirch, France, 2 Team « Evo-Devo of Vertebrate Dentition », Institut de Génomique Fonctionnelle de Lyon, Unité Mixte de Recherche 5242 Centre National de la Recherche Scientifique, Ecole Normale Supérieure de Lyon, Claude Bernard Lyon 1 University, Lyon, France, 3 Faculty of Dentistry, University of Strasbourg, Strasbourg France, 4 UF6237 Preclinical Imaging Lab, Biophysics and Nuclear Medicine, Hôpitaux Universitaires de Strasbourg (HUS), Strasbourg, France, 5 Reference Centre for Orodental Manifestations of Rare Diseases, Pôle de Médecine et Chirurgie Bucco-dentaires, Hôpitaux Universitaires de Strasbourg (HUS), Strasbourg, France, 6 INSERM U1121, "Biomaterials and Bioengineering", University of Strasbourg, Strasbourg, France, 7 Faculty of Dentistry, Khon Kaen University, Khon Kaen, Thailand

Abstract

Background: The RSK2 gene is responsible for Coffin-Lowry syndrome, an X-linked dominant genetic disorder causing mental retardation, skeletal growth delays, with craniofacial and digital abnormalities typically associated with this syndrome. Craniofacial and dental anomalies encountered in this rare disease have been poorly characterized.

Methodology/Principal Findings: We examined, using X-Ray microtomographic analysis, the variable craniofacial dysmorphism and dental anomalies present in Rsk2 knockout mice, a model of Coffin-Lowry syndrome, as well as in triple Rsk1,2,3 knockout mutants. We report Rsk mutation produces surpernumerary teeth midline/mesial to the first molar. This highly penetrant phenotype recapitulates more ancestral tooth structures lost with evolution. Most likely this leads to a reduction of the maxillary diastema. Abnormalities of molar shape were generally restricted to the mesial part of both upper and lower first molars (M1). Expression analysis of the four Rsk genes (Rsk1, 2, 3 and 4) was performed at various stages of odontogenesis in wild-type (WT) mice. Rsk2 is expressed in the mesenchymal, neural crest-derived compartment, correlating with proliferative areas of the developing teeth. This is consistent with RSK2 functioning in cell cycle control and growth regulation, functions potentially responsible for severe dental phenotypes. To uncover molecular pathways involved in the etiology of these defects, we performed a comparative transcriptomic (DNA microarray) analysis of mandibular wild-type versus Rsk2-/Y molars. We further demonstrated a misregulation of several critical genes, using a Rsk2 shRNA knock-down strategy in molar tooth germs cultured in vitro.

Conclusions: This study reveals RSK2 regulates craniofacial development including tooth development and patterning via novel transcriptional targets.

Introduction

The ribosomal S6 family of serine/threonine kinases is composed of 4 highly related members in mammals: RSK1 (human chromosome 3), RSK2 (RP56K13, Xp22.2-22p21.1), RSK3 (chromosome 6) and RSK4 (Xq21), which are 75% homologous and are implicated in several important cellular events including proliferation, differentiation, cellular stress response and apoptosis. Mutations in RSK2 cause X-linked Coffin-Lowry syndrome (OMIM #303600) characterized by psychomotor and growth retardation, with typical facial and digital abnormalities and progressive skeletal malformations like delayed bone development, spinal kyphosis/scoliosis, and sternum/rib protrusions (pectus carinatum) or depression (excavatum) [1,2,3]. Orodental findings include a high narrow palate, a midline lingual furrow, malocclusion, and large medial incisors (Fig. 1). Premature loss of the primary dentition was also observed, and wide spaced teeth and large medial incisors were reported [1,4,5].

RSKs are Ser/Thr protein kinases that act at the distal end of the Mitogen-Activated Protein Kinase/Extracellular signal-Regu-
Reactivated Kinase (MAPK/ERK) signalling pathway. The various RSK proteins are widely expressed, with many cell types expressing several members. RSKs are directly phosphorylated and activated by ERK1/2 in response to several growth factors [6]. In the cytosol, RSK proteins have been shown to phosphorylate substrates including GSK3, L1CAM, the Ras GEF-Sos, IkB, the p34cdc2-inhibitory kinase Myt1, the translation factors eEF2 and eIF4B, and the pro-apoptotic protein BAD [7,8]. Moreover, upon activation of the cytosolic RSKs translocate to the nucleus where they are thought to regulate gene expression through phosphorylation of transcription factors such as CREB1, ERα, Nurr77 and SRF, as well as histones [7]. The contribution of each RSK family member to the in vivo activation of most of these substrates is currently not well defined.

RSK orthologues have been identified in mouse, rat, chicken, Xenopus and Drosophila [9]. Rsk2 knockout mice display spatial learning and memory impairment [10] and develop a progressive osteopenia due to impaired osteoblast function [11]. Lack of phosphorylation of the transcription factor ATF4 is responsible for the skeletal abnormalities in Rsk2 knockout mice [10]. Murine Rsk2 is highly expressed during somitogenesis, suggesting skeletal and muscle growth and/or patterning roles, potentially leading to the numerous skeletal abnormalities encountered in the human syndrome [12]. It is interesting to note that Rsk2 shows specific developmental patterns of expression in the maxillary and the mandibular components of the first branchial arch [12], and that craniofacial and dental anomalies are present in the clinical synopsis of Coffin-Lowry syndrome.

The mouse dentition is a powerful and useful model to study the mechanisms leading to human dental anomalies, despite some intrinsic differences. The mouse has a monophyodont dentition that encompases only four permanently growing incisors and 12 molars separated by a diastema [13]. Most mutant mouse models for human syndromic and non-syndromic genetic disorders displaying dental defects mimicking human pathologic phenotypes [14], although some discrepancies have been reported [15]. In this study, we describe the craniofacial and orodental phenotype of Rsk2-/Y knockout mice, as well as triple Rsk1,2,3-/− mutants. By employing several additional approaches (expression analysis by in situ hybridization, comparative transcriptomic analysis, shRNA knock-down in tooth germ explants) we establish RSK2 regulates defined processes in odontogenesis and craniofacial growth.

Results

**Rsk2-/Y craniofacial phenotype**

The craniofacial phenotype was assessed by X-Ray microtomographic imaging and analyzed through euclidean distances measurement and comparison. This phenotype was variable, ranging from normality (mutant mice 702, 731) to obvious craniofacial dysmorphology (mutant mice 150, 700) (Fig. 2). Some mutant mice show an intermediate phenotype, with skulls smaller in length but not in width.

An overall skull length reduction from the nasal to the occipital bone was a collective measure of the sum defects in the nasal, frontal, parietal, interparietal/occipital bones. The frontal bone always exhibited the greatest reductions in length of all skull bones. The interparietal/occipital bones were the least affected (distances 1–4, 1–5, 2–3, 2–4, 2–5 being more reduced than 1–2 or 1–3: see Table 1; measurements were performed according to anatomical landmarks detailed in Fig. S1). A nasal deviation was observed in some mutant mice (Fig. 2B,C,E; Table 1). Craniofacial microtomographic analysis of Rsk1,2,3−/− mice was not performed, due to rare availability of these triple mutants. Analysis thus focused on Rsk2−/Y mice, a defined genetic model of human Coffin-Lowry syndrome.

**Rsk2−/Y and Rsk1,2,3−/− dental phenotype**

**Phenotype of molar tooth rows.** We analyzed 15 Rsk2−/Y mice and 9 Rsk1,2,3−/− mutant mice (see Table 2 and Fig. 3). Supernumerary teeth (ST) occurred in all specimens except for two Rsk2−/Y mice (702, 731). ST were aligned with the molar tooth row and located just in front and at the contact of the first molars (M1).
The phenotype penetrance was higher in the upper dentition (65% in Rsk2-/Y mice, 83% in Rsk1,2,3-/− mice) than in the lower dentition (31% and 11%, respectively). Shape and size of the supernumerary teeth were variable and ranged from a monocuspid tooth to a well-shaped molar-like tooth. ST were always smaller in size than the adjacent first molars, and they were sometimes, especially in the Rsk1,2,3-/− upper rows, as big as the M2. Inactivation of Rsk2 alone was sufficient to generate the phenotype. When a ST was present, the mesial part of the first molar displayed altered crown morphology (Fig. 3). Even when the phenotype looked almost normal, a significant reduction in M1-M3 length was observed (Table 3). When ST occurred, the length mean value was always reduced for both M1 and M2 (Table 3). ST were always longer than M3. Molar width remained stable for M1, but was reduced for M2 and M3, except when only 3 molars were present in the mandible (Table 4). Shape abnormalities mainly occurred in mesial parts of the first lower (M1) and first upper (M1) molars in Rsk2-/Y mice. In upper M1, the central cusp of the mesial crest tended to be reduced, and the crest itself was more flattened (see red dotted circles in Fig. 3). Accordingly the mesial crest of the lower M1 also had an abnormal shape, because this crest tended to be strongly reduced when a ST occurred (see yellow dotted circles in Fig. 3). In triple mutants without ST (e.g. 789), the lower M1 displayed a mesial crest with vestibular cusp bigger than in WT.

Abnormal molar root development in Rsk2-/Y mice. Both the number and shape of molar roots also reflect the tooth identity. In WT mice the mandibular molars M1 and M2 have 2 roots, whereas M3 has only one root. The maxillary molars M1 and M2 have 3 roots. Variations in the root number of M3 occur both within and among various WT strains. The upper M3 molar usually has 2 or 3 roots, although sometimes it has only 1 root. Thus, the total number of molar roots is 5 per hemi-mandible, whereas it can vary from 7 to 9 per hemi-maxillary. In Rsk2-/Y mice, hemi-mandibles harbouring a ST had a total root number of 6, with the ST always having a single root. In the

| Distances          | Mean WT | STD WT | Mean Rsk2 mutants | STD Rsk2 mutants | p-value |
|--------------------|---------|--------|-------------------|------------------|---------|
| Between 1 and 2    | 7.10    | 0.59   | 6.84              | 0.35             | 0.03    |
| Between 1 and 3    | 15.05   | 0.46   | 13.92             | 0.59             | 0.01    |
| Between 1 and 4    | 18.59   | 0.41   | 17.24             | 0.57             | 0.005   |
| Between 1 and 5    | 21.87   | 0.49   | 20.48             | 0.48             | 0.005   |
| Between 2 and 3    | 8.07    | 0.45   | 7.25              | 0.47             | 0.005   |
| Between 2 and 4    | 11.72   | 0.38   | 10.69             | 0.45             | 0.005   |
| Between 2 and 5    | 15.21   | 0.35   | 14.13             | 0.31             | 0.005   |
| Between L and R 6  | 12.08   | 0.17   | 12.16             | 0.13             | 0.47    |
| Between L and R 7  | 6.42    | 0.21   | 6.45              | 0.27             | 0.68    |
| Between L and R 8  | 4.77    | 0.11   | 4.79              | 0.73             | 0.22    |
| Between L and R 9  | 6.27    | 0.25   | 6.13              | 0.12             | 0.06    |
| Between L and R 10 | 0.74    | 0.06   | 0.71              | 0.08             | 0.47    |
| Between L and R 11 | 1.05    | 0.05   | 1.13              | 0.11             | 0.29    |
| Between L and R 12 | 10.08   | 0.37   | 10.22             | 0.28             | 0.29    |
| Between L and R 15 | 4.53    | 0.19   | 4.72              | 0.51             | 0.37    |
| Between L and R 16 | 2.29    | 0.22   | 2.23              | 0.23             | 0.57    |
| Between L and R 6R | 15.24   | 0.34   | 14.39             | 0.52             | 0.008   |
| Between L and R 6L | 15.20   | 0.32   | 14.47             | 0.31             | 0.008   |
| Between L and R 7R | 7.92    | 0.28   | 7.18              | 0.58             | 0.008   |
| Between L and R 7L | 7.89    | 0.22   | 7.24              | 0.34             | 0.005   |
| Between L and R 8R | 6.90    | 0.24   | 6.29              | 0.46             | 0.88    |
| Between L and R 8L | 6.90    | 0.25   | 6.35              | 0.29             | 0.68    |
| Between L and R 9R | 20.65   | 0.47   | 19.55             | 0.46             | 0.008   |
| Between L and R 9L | 20.62   | 0.48   | 19.56             | 0.32             | 0.008   |

In bold are distances significantly different between wild- type (WT) and mutants (all these distances were shorter in mutants). Anatomical landmarks are explained in Fig. S1 (supplementary information). All distances are in millimeters. STD: standard deviation; L: left; R: right.

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The phenotype penetrance was higher in the upper dentition (65% in Rsk2-/Y mice, 83% in Rsk1,2,3-/− mice) than in the lower dentition (31% and 11%, respectively). Shape and size of the supernumerary teeth were variable and ranged from a monocuspid tooth to a well-shaped molar-like tooth. ST were always smaller in size than the adjacent first molars, and they were sometimes, especially in the Rsk1,2,3-/− upper rows, as big as the M2. Inactivation of Rsk2 alone was sufficient to generate the phenotype. When a ST was present, the mesial part of the first molar displayed altered crown morphology (Fig. 3). Even when the phenotype looked almost normal, a significant reduction in M1-M3 length was observed (Table 3). When ST occurred, the length mean value was always reduced for both M1 and M2 (Table 3). ST were always longer than M3. Molar width remained stable for M1, but was reduced for M2 and M3, except when only 3 molars were present in the mandible (Table 4). Shape abnormalities mainly occurred in mesial parts of the first lower (M1) and first upper (M1) molars in Rsk2-/Y mice. In upper M1, the central cusp of the mesial crest tended to be reduced, and the crest itself was more flattened (see red dotted circles in Fig. 3). Accordingly the mesial crest of the lower M1 also had an abnormal shape, because this crest tended to be strongly reduced when a ST occurred (see yellow dotted circles in Fig. 3). In triple mutants without ST (e.g. 789), the lower M1 displayed a mesial crest with vestibular cusp bigger than in WT.

Incisor phenotype. No statistically significant differences were revealed between right and left incisors in both groups (p>0.05). As a consequence, results from right and left incisors were merged (Table 5). No statistical difference was observed between WT and Rsk2-/Y (p>0.05). No obvious incisor anomaly was detected. Analysis of the incisor phenotype in Rsk1,2,3-/− mice was not performed due to a reduced number of available animals, visual inspection however did not revealed any incisor dental defects. The existence of incisor anomalies in Rsk1,2,3-/− cannot be ruled out.

Abnormal molar root development in Rsk2-/Y mice. Both the number and shape of molar roots also reflect the tooth identity. In WT mice the mandibular molars M1 and M2 have 2 roots, whereas M3 has only one root. The maxillary molars M1 and M2 have 3 roots. Variations in the root number of M3 occur both within and among various WT strains. The upper M3 molar usually has 2 or 3 roots, although sometimes it has only 1 root. Thus, the total number of molar roots is 5 per hemi-mandible, whereas it can vary from 7 to 9 per hemi-maxillary. In Rsk2-/Y mice, hemi-mandibles harbouring a ST had a total root number of 6, with the ST always having a single root. In the
maxillary quadrant, the total root number varied from 8 to 9 when a ST was present. ST could have 1 or 2 roots, M1 had 2 roots, but the M2 root number remained stable with 3 roots (Table S1 in File S1). The presence of maxillary M1 with only 2 roots probably correlates with the occurrence of ST, often associated to a compression and a reduction of the mesial part of M1.

**Rsk2-/Y and the diastema.** The maxillary diastema was significantly reduced in Rsk2-/Y mice harbouring ST (Table 6). This was not the case for the mandibulary diastema, whatever the number of teeth.

**Expression patterns of Rsk genes during mouse odontogenesis**

To investigate whether the dental anomalies observed in the Rsk-deficient mice may correlate with distinct patterns of expression of Rsk genes during odontogenesis, we performed an in situ hybridization analysis of Rsk1, Rsk2, Rsk3 and Rsk4 mRNA transcripts at various stages of tooth development. All four genes were found to be expressed in specific areas of the tooth anlagen, as illustrated in Fig. 4 for the developing first molars, and Fig. 5 for the mandibular incisors. Their expression features are summarized in Table 7.

Rsk2 transcripts were mainly localized in the mesenchymal compartment or dental papilla from E12.5 (dental lamina stage) to E19.5 (late bell stage). At E16.5 (bell stage), the transcripts were scattered in the area facing the epithelial loops or at the base of the cusps; the second molar dental mesenchyme was uniformly labelled. In the incisors the transcripts were also located in the mesenchyme, and the signal was more intense in the labial area and posterior area.

The expression of Rsk1 and Rsk4 was mainly epithelial, marking the inner dental epithelium and especially the epithelial loop areas. A faint signal was also observed for Rsk4 in the posterior mesenchymal area facing the epithelial loops especially the labial loop for incisors (E16.5 and 19.5).

Rsk3 transcripts were mainly visible in the dental papilla at E14.5 (cap stage), E16.5 (bell stage) and E19.5 (Figs. 4, 5 and Table 7). At E19.5 the transcripts were concentrated in the cervical part of the papilla for the first molar and had a wider distribution in the second molar mesenchyme.

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**Figure 3. Variation of molar shape, size and number in Rsk2-/Y and Rsk1,2,3-/ mice analyzed by X-Ray microtomography.** All molar rows are oriented in the same manner (top corresponds to mesial and left to lingual side). At the left are wild-type molars, other rows are mutants as indicated. Arrowheads point to the supernumerary teeth ST; dotted ellipses show the reduction of the mesial-most affected cusp. Scale bar: 0.7 μm.

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Rsk2-/Y transcriptome analysis

We performed a comparative transcriptomic analysis (Affymetrix GeneChips) of wild-type and Rsk2-/Y mice, analyzing RNA isolated from dissected E14.5 mandibular molar tooth germs. By using a principal component analysis (PCA), it was possible to discriminate between the molar samples according to their genotype (wild-type versus Rsk2-/Y) (Fig. 7). 504 genes were retrieved as being differentially expressed, with fold changes exceeding ±1.2 and a false discovery rate below 0.1. The fold changes were however rather low, ranking between +2.044 and -1.98, suggesting that rather minimal molecular changes, at the transcriptome level, were taking place. These changes could be also minimized by a heterogeneous sampling for Rsk2-/Y molars, with putative phenotypes ranking as previously described between normality and a 3 molars with a supernumerary tooth phenotype; this phenotype was not known at the E14.5 cap stage, when molar germs were pooled for transcriptomic analysis. The most affected (“top ten”) genes, and a selection of additional genes being up- or down-regulated, are listed in Table 8 and Table 9.

Table 2. Presence (+) of a supernumerary tooth (mesial to the first molar) in Rsk mutant mice.

| Mouse | Genotype | Upper Right | Upper Left | Lower Right | Lower Left |
|-------|----------|-------------|------------|-------------|------------|
| 11    | Rsk2-/Y  | + (4)       | + (4)      | + (4)       | N          |
| 25    | Rsk2-/Y  | + (4)       | + (4)      | N           | + (4)      |
| 37    | Rsk2-/Y  | + (4)       | + (4)      | + (4)       | N          |
| 39    | Rsk2-/Y  | + (4)       | + (4)      | N           | N          |
| 46    | Rsk2-/Y  | + (4)       | N          | N           | N          |
| 61    | Rsk2-/Y  | + (4)       | + (4)      | N           | N          |
| 62    | Rsk2-/Y  | N           | + (4)      | N           | N          |
| 150   | Rsk2-/Y  | + (4)       | + (4)      | + (4)       | + (4)      |
| 155   | Rsk2-/Y  | + (4)       | + (4)      | + (4)       | + (4)      |
| 184   | Rsk2-/Y  | + (4)       | + (4)      | + (4)       | + (4)      |
| 185   | Rsk2-/Y  | + (4)       | + (4)      | N           | N          |
| 700   | Rsk2-/Y  | N           | N          | N           | + (4)      |
| 702   | Rsk2-/Y  | N           | N          | N           | N          |
| 728   | Rsk2-/Y  | + (4)       | N          | N           | N          |
| 731   | Rsk2-/Y  | N           | N          | N           | N          |
| 789   | Rsk1,2,3/- | + (4)     | + (4)      | N           | N          |
| 793   | Rsk1,2,3/- | + (4)     | N          | N           | N          |
| 860   | Rsk1,2,3/- | + (4)     | N          | + (4)       | N          |
| 883   | Rsk1,2,3/- | + (4)     | + (3)      | N           | N          |
| 1105  | Rsk1,2,3/- | + (4)     | N          | N           | N          |
| 1111  | Rsk1,2,3/- | + (3)     | + (3)      | + (4)       | N          |
| 1283  | Rsk1,2,3/- | + (3)     | + (4)      | N           | N          |
| 1139  | Rsk1,2,3/- | + (3)     | + (4)      | N           | N          |
| 1159  | Rsk1,2,3/- | + (3)     | + (3)      | N           | N          |

N: normal pattern of 3 molars M1, M2, M3. (3) means the presence of only 3 molars but the size of the second one is bigger (most probably there is a supernumerary tooth ST in front of M1, and M3 is missing).

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Rsk2-/Y supernumerary tooth is associated with an extra mesial placode

An extra tooth placode, and subsequent enamel knot labelled with Sonic hedgehog (Shh) as a marker, was clearly visible at E14.5, mesially to M1 in Rsk2-/Y mice when compared to WT littermates (Fig. 6). The presence of this extra placode was seen in nearly 30% of the analysed embryo and was not always simultaneously present on right and left sides of the jaw. During WT development the three molars (M1, M2 and M3) are forming from a unique molar placode. The presence of an additional dental placode mesially to the M1 placode likely corresponds to the primordium of ST developing in Rsk2-/Y mutants.

Rsk2-/Y inactivation in vivo

In order to overcome the potential problem of Rsk2-/Y molar samples heterogeneity we decided to use an in vitro system, and inactivated Rsk2 by microinjection and electroporation of shRNA in E14.5 molar tooth germs. In vivo electroporation in the mandible, as previously described by [23] was not suitable for our study, because of the lack of precision on the targeted zone and our aim to inactivate the gene directly within the tooth anlagen.

We optimized the experimental settings to inject and electro- porate Rsk2 shRNA in cap stage molars explanted and cultured ex vivo. Control explants were electroporated with a random shRNA construct. After electroporation the explants were placed into collagen drop culture to maintain their morphology [24] and kept in a defined culture medium for 24 h, before being processed for qRT-PCR analysis. Tooth morphology was preserved and neither apoptosis nor cell death were induced, as shown by activated caspase3 and TUNEL assays (data not shown).

Rsk2 shRNA electroporation efficiently diminished Rsk2 expression by 75% (Fig. 8A). We then analyzed Eaf2 and Rodh1 expression, and found that Eaf2 expression was down-regulated (75% decrease) (Fig. 8B), while Rodh1 expression was up-regulated (50% increase)(Fig. 8C). These results were in accordance with the microarray data. The new combined technique allowed us not only to optimize a protocol for studying the molecular mechanisms of early tooth development, but also to set up a new approach for microarray validation.

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A peculiar dental phenotype

Mice are commonly studied mammals for investigating the mechanisms of tooth development and evolution. The orodental phenotype of mouse models of human genetic disorders also often mimics dental anomalies encountered in human rare diseases [14]. Basal placental mammals had a dental formula including three incisors, one canine, four premolars, and three molars per dentition quadrant [13,25]. Through evolution, almost all

Table 3. Comparison of each molar length, of the total molar field length, and the total length of M1+ M2, between wild-type (WT) mice and Rsk2-/-Y mutant mice with 3 or 4 molars.

|                      | Mandibular molars WT | Mandibular molars Mutant 3M | Mandibular molars Mutant 4M | Maxillary molars WT | Maxillary molars Mutant 3M | Maxillary molars Mutant 4M |
|----------------------|----------------------|-----------------------------|-----------------------------|---------------------|-----------------------------|-----------------------------|
| Mean ST              | 1.44                 | 0.60                        | 0.74                        | 1.38                | 0.74                        | 1.14                        |
| STD                  | 0.01                 | 0.01                        | 0.02                        | 0.09                | 0.02                        | 0.02                        |
| Mean M1              | 1.14                 | 1.14                        | 1.56                        | 1.38                | 1.14                        | 1.14                        |
| STD                  | 0.07                 | 0.07                        | 0.07                        | 0.10                | 0.09                        | 0.09                        |
| p-value              | 0.0004               | 0.0004                      | 0.0004                      | 0.001               | 0.0001                      | 0.0001                      |
| Mean M2              | 1.60                 | 0.89                        | 0.85                        | 0.92                | 0.85                        | 0.82                        |
| STD                  | 0.02                 | 0.02                        | 0.02                        | 0.02                | 0.02                        | 0.02                        |
| p-value              | 0.0004               | 0.0004                      | 0.0004                      | 0.001               | 0.0001                      | 0.0001                      |
| Mean M3              | 2.60                 | 0.59                        | 0.53                        | 0.60                | 0.63                        | 0.63                        |
| STD                  | 0.02                 | 0.03                        | 0.03                        | 0.03                | 0.05                        | 0.05                        |
| p-value              | 0.0002               | 0.0002                      | 0.0002                      | 0.001               | 0.0001                      | 0.0001                      |
| Total                | 2.93                 | 2.75                        | 2.88                        | 3.08                | 2.85                        | 3.31                        |
| STD                  | 0.04                 | 0.15                        | 0.28                        | 0.10                | 0.13                        | 0.06                        |
| p-value              | 0.0008               | 0.0004                      | 0.0004                      | 0.01                | 0.0001                      | 0.0001                      |
| M1+ M2               | 2.33                 | 2.10                        | 2.15                        | 2.48                | 2.30                        | 1.96                        |
| STD                  | 0.04                 | 0.16                        | 0.21                        | 0.08                | 0.14                        | 0.13                        |
| p-value              | 0.03                 | 0.04                        | 0.04                        | 0.03                | 0.05                        | 0.05                        |

M1 size is reduced in mutants with 4 molars both in mandible and maxilla, but only in the maxilla of mutants showing 3 molars. M2 is reduced both in maxilla and mandible whatever the molar mutant phenotype. M3 mesiodistal dimension is reduced only in the mandible, irrespective of the presence of 3 or 4 molars. The total size of the molar field is reduced in the mandible and maxilla in mutant with 3 molars. The total size of the molar field is significantly increased in the maxilla in mutants with 4 molars. The total size of M1+M2 was always reduced when compared to the wild type value. All distances are in millimeters. Significant p-values are shown in bold. M1 : molar 1; M2 : molar 2; M3 : molar 3; ST : supplementary tooth in front of M1; STD : standard deviation; Mutant 3M : mutant with 3 molars; Mutant 4M : mutant with 4 molars; Total : total length of all molars in the molar field.

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Discussion

A peculiar dental phenotype

Mice are commonly studied mammals for investigating the mechanisms of tooth development and evolution. The orodental phenotype of mouse models of human genetic disorders also often mimics dental anomalies encountered in human rare diseases [14]. Basal placental mammals had a dental formula including three incisors, one canine, four premolars, and three molars per dentition quadrant [13,25]. Through evolution, almost all

Table 4. Comparison of each molar width (vestibulolingual dimension) between wild-type and Rsk2-/-Y mutant mice with 3 or 4 molars.

|                      | Mandibular molars WT | Mandibular molars Mutant 3M | Mandibular molars Mutant 4M | Maxillary molars WT | Maxillary molars Mutant 3M | Maxillary molars Mutant 4M |
|----------------------|----------------------|-----------------------------|-----------------------------|---------------------|-----------------------------|-----------------------------|
| Mean ST              | 0.59                 | 0.76                        | 0.76                        | 0.09                | 0.04                        | 0.04                        |
| STD                  | 0.09                 | 1.04                        | 1.04                        | 0.05                | 0.05                        | 0.05                        |
| Mean M1              | 0.79                 | 0.82                        | 0.81                        | 0.90                | 0.86                        | 0.86                        |
| STD                  | 0.03                 | 0.03                        | 0.03                        | 0.05                | 0.05                        | 0.05                        |
| p-value              | 0.02                 | 0.02                        | 0.02                        | 0.02                | 0.02                        | 0.02                        |
| Mean M2              | 0.85                 | 0.85                        | 0.81                        | 0.90                | 0.86                        | 0.86                        |
| STD                  | 0.02                 | 0.02                        | 0.02                        | 0.02                | 0.02                        | 0.02                        |
| p-value              | 0.06                 | 0.06                        | 0.06                        | 0.06                | 0.06                        | 0.06                        |

In mutants the width of M1 was not modified. The width of the maxillary M2 and M3 was reduced. In the mandible the width of M2 was lower only when 4 molars were present, and the width of M3 was lower only in mutants with 3 molars (all distances in millimeters; significant p-values in bold). M1 : molar 1; M2 : molar 2; M3 : molar 3; ST : supplementary tooth in front of M1; STD : standard deviation; Mutant 3M : mutant with 3 molars; Mutant 4M : mutant with 4 molars.

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Rsk2 phenotype is similar to the tabby phenotype

Supernumerary teeth within the diastema were described in other mouse mutants for ectodysplasin A (Eda; also known as Ta; Ed1; EDA1; XLHED; tabby) and Eda receptor (Edar) [39,40,41,42]. The phenotype in the light of Coffin-Lowry syndrome include a thick calvarium, hyperplastic maxillary, mammary glands and tooth; OMIM #305100). Mutations in the human X-linked gene ED1 (the Eda orthologue) are responsible for this disorder [43,44,45,46]. Ontogenetic and phylogenetic data together suggest that the occurrence of functional supernumerary teeth in tabby mice diastema is caused by the persistence of the premolar rudiments that usually regress in WT mice. It has thus been proposed that this phenotype may represent an evolutionary throwback or atavism [43,44,45,46].

Recently is has been shown that the number of teeth is regulated by fine-tuning of the level of receptor-tyrosine kinase signalling [32]. Known cytosolic RSK substrates include I-kappaB alpha [7,47,48], a member of the NF-kappaB pathway. This pathway is involved in development and in some human disorders including ectodermal dysplasias that affect ectodermal derivatives like the teeth, hair, salivary, mammary glands and skin. I-kappaB alpha has specifically been associated with an autosomal dominant form of anhidrotic ectodermal dysplasia and with T cell immunodeficiency [49]. The fact that I-kappaB alpha can be phosphorylated by RSKs, leading to increase of NF-kappaB activity, may therefore explain some of the dental anomalies encountered in the Rsk2-/Y transgenic mice and in Coffin-Lowry syndrome patients. However, the downstream signalling pathway(s) of Eda/Edar involved in tooth formation in the diastema are not yet known.

The phenotype in the light of Coffin-Lowry syndrome

The mouse model for Coffin-Lowry syndrome, obtained by inactivation of the Rsk2 gene and used in this study, was previously described by [11]. Mutant mice weigh 10% less and are 14% shorter than their wild-type littermates. This is in agreement with a size of the skull could be associated to the general growth defect. Recently is has been shown that the number of teeth is regulated by fine-tuning of the level of receptor-tyrosine kinase signalling [32]. Known cytosolic RSK substrates include I-kappaB alpha [7,47,48], a member of the NF-kappaB pathway. This pathway is involved in development and in some human disorders including ectodermal dysplasias that affect ectodermal derivatives like the teeth, hair, salivary, mammary glands and skin. I-kappaB alpha has specifically been associated with an autosomal dominant form of anhidrotic ectodermal dysplasia and with T cell immunodeficiency [49]. The fact that I-kappaB alpha can be phosphorylated by RSKs, leading to increase of NF-kappaB activity, may therefore explain some of the dental anomalies encountered in the Rsk2-/Y transgenic mice and in Coffin-Lowry syndrome patients. However, the downstream signalling pathway(s) of Eda/Edar involved in tooth formation in the diastema are not yet known.

| Table 5. Morphological analysis of mandibular incisors in WT and Rsk2-/Y mice. |
|-------------------------------------------------|
| **Li (mm)** | **hi (mm)** | **Hl (mm)** | **li (mm)** | **ti (mm)** | **lpa (mm²)** |
| WT Mean | 11.33 | 1.11 | 2.96 | 9.38 | 0.65 | 10.36 |
| STD | 2.34 | 0.07 | 0.20 | 1.56 | 0.02 | 1.07 |
| Rsk2-/Y Mean | 11.29 | 1.14 | 2.97 | 9.05 | 0.65 | 10.18 |
| STD | 1.66 | 0.05 | 0.21 | 1.65 | 0.03 | 1.33 |

No statistically significant differences were revealed between right and left incisors in both groups (p > 0.05). As a consequence, results from right and left incisors were merged. No statistical difference was observed between WT and Rsk2-/Y (p > 0.05).

The dental phenotype of the Rsk2-/Y mouse resembles that encountered in the tabby mouse (a model for X-linked hypohidrotic ectodermal dysplasia- an ectodermal dysplasia characterized by an absence or dystrophic development of hair, nail, sweat-lacrimal-mammary- glands and tooth; OMIM #305100). Ontogenetic and phylogenetic data together suggest that the occurrence of functional supernumerary teeth in tabby mice diastema is caused by the persistence of the premolar rudiments that usually regress in WT mice. It has thus been proposed that this phenotype may represent an evolutionary throwback or atavism [43,44,45,46].

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| Table 6. Upper and lower diastema size in wild-type and Rsk2-/Y mutant mice with 3 or 4 molars. |
|-------------------------------------------------|
| **Mean size (mm)** | **STD** | **p-value** |
| Mandibular diastema Wild-type | 2.99 | 0.09 |
| Mandibular diastema Mutants with 3 molars | 2.83 | 0.21 | 0.22 |
| Mandibular diastema Mutants with 4 molars | 2.71 | 0.1 | 0.26 |
| Maxillary diastema Wild-type | 5.45 | 0.18 |
| Maxillary diastema Mutants with 3 molars | 5.33 | 0.21 | 0.49 |
| Maxillary diastema Mutants with 4 molars | 5.01 | 0.21 | 0.0019 |

Only the upper (maxillary) diastema from mutants with 4 molars was significantly smaller compared to wild-type (bold). STD : standard deviation.
defects, numerous supernumerary teeth, and delayed tooth eruption. The mouse model of this disease (Cbfal-KO) exhibits arrested tooth development [15]. Mostly loss of function mutations in RSK2 both in human and mouse are responsible for the phenotype. Despite striking similarities mouse and human tooth development, cross-species comparison of mouse/human denti-

Figure 4. Expression patterns of Rsk genes during mouse molar odontogenesis at E12.5 (dental lamina), E14.5 (cap), E16.5 (bell) and E19.5 (late bell) stages, analyzed by in situ hybridization. Abbreviations: DL dental lamina, DP dental papilla, EL epithelial loop, EO enamel organ, IDE inner dental epithelium, M1 first molar, M2 second molar, Mes ectomesenchyme, ODE outer dental epithelium, PreOd preodontoblasts, To tongue. Scale bars 140 μm (A,B,C,D,I,J,K and L) and 250 μm (E,F,G,H,M,N,O and P). doi:10.1371/journal.pone.0084343.g004

Figure 5. Expression patterns of Rsk genes during incisor odontogenesis at E12.5 (dental lamina), E14.5 (cap), E16.5 (bell) and E19.5 (late bell) stages analyzed by in situ hybridization. Abbreviations: DL dental lamina, DP dental papilla, EL epithelial loop, EO enamel organ, IDE inner dental epithelium, LaEL labial epithelial loop, LIEL lingual epithelial loop, Mes ectomesenchyme, PreAm preameloblasts. Scale bars 140 μm (A,B and C), 200 μm (D,E, F,G, J, K and L), 250 μm (M,N,O and P) and 50 μm (H). doi:10.1371/journal.pone.0084343.g005
tions show they are clearly not alike. This suggests differential output and involvement of identical or superimposed pathways. These differences may account for this apparently opposite in nature dental phenotype i.e. hypodontia in human versus supernumerary teeth in mouse.

Cell cycle progression and cell proliferation

It has been shown that tooth number, size, and shape are linked to cell proliferation and apoptosis events [50,51,52,53]. It is interesting to note that Rsk1 genes are expressed throughout tooth development, especially in proliferating areas (this study, Figs. 4 and 5), either in the mesenchyme (Rsk2, Rsk3) or in the epithelial loops (Rsk1, Rsk4). The expression patterns described for the Rsk genes in other embryonic regions [12,54,55] also indicate a correlation with proliferative areas. Rsk1 is highly expressed in tissues harbouring highly proliferating cells like liver, lung, thymus, and olfactory and respiratory epithelia. Particularly intense Rsk1 expression is observed in the gut epithelium. Rsk2 is expressed at very low levels throughout mouse development in dorsal root ganglia, thyroid gland, kidney, lung, liver and skeletal muscles. Rsk3 is mainly expressed in the nervous system, but also in the

Figure 6. Whole mount in situ hybridization showing the molar and incisor placodes within the mandible at E14.5 in a wild-type mouse (A) and Rsk2-/- mouse (B: detail of the right-side mandible) labelled with a Shh riboprobe. Two molar placodes are clearly visible in B (arrows) Scale bars 200 μm (A), 100 μm (B).

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| E12.5 | E14.5 | E16.5 | E19.5 |
|---|---|---|---|
| **Rsk1** | **Rsk2** | **Rsk3** | **Rsk4** |
| Inc | M1 | M2 | M3 | Inc | M1 | M2 | M3 | Inc | M1 | M2 | M3 |
| Oral epithelium | + | + | + | + | + | + |
| Dental lamina | + | + | + faint | + | + | + |
| Mesenchyme | + | + | + | + |
| Enamel organ | + | + | + | + |
| IDE | + | + | + | + |
| Enamel knot | + π faint | + | + | + |
| Dental papilla | + lab | + lab | + | + | + + | + + |
| Dental sac | + |

+ : positive signal; lab: labial; π posterior area
IDE: inner dental epithelium; ODE: outer dental epithelium; SR: stellate reticulum; SI: stratum intermedium; PreAm: preameloblasts; Am: ameloblasts; Od: odontoblasts, PreOd: Preadontoblasts
doi:10.1371/journal.pone.0084343.t007
RSK2 and Craniofacial Development

Increased proliferation and decreased apoptosis are necessary to develop supernumerary teeth, as seen by differential regulation of Sprouty genes [57]. The localisation of Rsk3 genes in proliferative areas, their role in the regulation of proliferation and apoptosis [58] as well as the interconnection of the involved pathways could explain that the deletion of Rsk2 gene could induce the formation of supernumerary teeth and therefore reveal ancestral stages of murine dental evolution.

RSK and the Ras/MAPK pathway

Altered ERK/MAPK signalling (with abnormally increased phosphorylation of ERK1/2) was recently reported in the hippocampus of the Rsk2-/Y mice [59]. RSK2 was also proven to exert a feedback inhibitory effect on the ERK1/2 pathway. Our microarray data did not indicate a similar trend at the level of the developing molar. Ngf was the only gene detected by our microarray analysis, known to be regulated at the transcriptional level by ERK1/2 and involved in tooth development [60].

Unravelling target genes and involved pathways

The heterogeneity of the molar samples and the variability of the Rsk2-/Y molar phenotype has certainly interfered with the analysis of the microarray data and may have prevented the confirmation of target gene expression by qRT-PCR. However, genes involved in TGF-beta receptor signalling (Amhr2), or in the FGF (Fgfbp3) and Wnt (Sfrp5) pathways, were shown to be affected by the inactivation of Rsk2. These pathways are known to be involved in tooth development [61] and, when affected, are responsible for dental anomalies both in mouse models and human [62]. The in vitro assay we have developed, involving micro-injection and electroporation of a Rsk2 shRNA in explanted molar tooth germs, proved to be a valuable system to assess quantitative changes in target gene expression.

Conclusions

Analysis of Rsk2-/Y mutant mice revealed an important role of Rsk2 in craniofacial development, especially in dentition development and patterning. The loss of function of Rsk2 allows the reappearance of supernumerary diastemal teeth considered as remnants of teeth lost over evolution. Our in situ hybridization analysis indicated that Rsk2 expression pattern may correlate with proliferative areas of the developing teeth, consistent with a biological function of RSK2 in cell-cycle control and cell growth. Transcriptome profiling analysis was difficult because of the heterogeneity and variability of the Rsk2-/Y molar phenotype has certainly interfered with the analysis of the microarray data did not indicate a similar trend at the level of the developing molar. Ngf was the only gene detected by our microarray analysis, known to be regulated at the transcriptional level by ERK1/2 and involved in tooth development [60].

Materials and Methods

Patients and Ethics Statement

Patients attending the Reference centre for orodental manifestations of rare diseases, Pôle de médecine et chirurgie bucco-dentaire, Hôpitaux Universitaires de Strasbourg, or their legal representatives (parents, next of kin, caretakers or guardians) if minor and/or not in capacity to provide his or her own consent, gave written informed consent for the use and publication of medical data. The picture proposed as figure 1 was uploaded in D4/phenodent database (CCTIRS positive report 11/09/2008, CNIL authorization 18/05/2008).
Animals and Ethics Statement

Rsk2 gene targeting was previously described [11]. As in human, the mouse Rsk2 gene is located on the X chromosome. Analyses were performed on Rsk2-/Y ("knockout") male mice, with littermate wild-type (Rsk2+/Y) males used as control animals. Triple Rsk1,2,3-/- mutant mice were obtained by breeding of single knockout mice, themselves obtained by excision of exons three and four (RSK1 knockout) or exon two (RSK2 and RSK3 knockouts) of the corresponding genes, leading to frameshift mutations [11]. The experiments were performed in accordance with the French national and European Laws and Directives Concerning Laboratory Animal Housing, Welfare and Experimentation and after approval from the CERBM-GIE: ICS/IGBMC Ethical Research Board.

X-Ray microtomography

A cohort of 6 mutant Rsk2-/Y male mice (#150, 155, 700, 702, 728, 731) and 6 wild-type (WT) littermates (#149, 154, 699, 727, 729, 730), as well as 7 Rsk1,2,3 compound mutant male mice (#789, 860, 883, 1105, 1111, 1199, 1283), were analyzed through X-ray micro-computed tomography (micro-CT) (GE eXplore Vision CT120 (General Electric, Waukesha, USA) for craniofacial exploration and Phoenix Nanotom (General Electric measurement and control, Billerica, USA) for tooth analysis). For craniofacial exploration the micro-CT was performed using 220 projections with an angle increment of 0.877 degrees (Parker mode) and one average frame per projection (70.0 kV, 32 mA, and exposition time of 16 milliseconds). 3D-reconstructions were done using a Feldkamp algorithm giving voxels of 100 x 100 x 100 μm³. For teeth a second micro-CT acquisition was performed with a smaller reconstructed voxel size (3 x 3 x 3 μm³) using 2 000 projections and three average frames per projection (100.0 kV, 70 μA, and exposure time of 500 ms). 3D-reconstructions were done using datos|x software and algorithm including beam hardening correction and ring artefact reduction.

Anatomical landmarks were identified as remarkable points (for instance cranial sutures) that could be easily recognized on each specimen. Fig. S1 (supplementary material) illustrates this step, showing on a 3D isosurface rendering the landmarks (red points)
Calculation involved euclidean distances between selected pairs of points that could be compared to identify statistical significant differences between WT and mutant mice. The methodology is based upon previously described work [63,64]. To avoid any bias in landmarks position, landmarks were placed two times by one investigator (intra-investigator reproducibility) and one time by another investigator (inter-investigator reproducibility).

Table 9. Overview of other genes differentially expressed in Rsk2-/Y and wild-type developing mandibular molars.

| Up-regulated genes | Fold change | P-value | Name |
|--------------------|-------------|---------|------|
| Mdm2              | 1.42143     | 6.43E-07| p53 E3 ubiquitin protein ligase homolog oncoprotein |
| Neurog3           | 1.39695     | 4.46E-08| neurogenin 3 |
| Pou5f2            | 1.38695     | 2.35E-07| POU domain class 6 transcription factor 2 |
| Dfla              | 1.38633     | 4.93E-10| DNA fragmentation factor alpha subunit |
| Bdkrb1            | 1.38509     | 4.15E-08| bradykinin receptor beta 1 |
| Ofd1              | 1.37103     | 4.33E-08| oral-facial-digital syndrome 1 gene homolog; ciliogenic protein |
| Zfp759            | 1.35236     | 5.23E-07| zinc finger protein 759 |
| Cllar             | 1.32394     | 7.32E-07| CASP8 and FADD-like apoptosis regulator |
| Aa2k1             | 1.23472     | 3.15E-06| AP2 associated kinase 1 positive regulation of Notch signaling pathway |

| Down-regulated genes | Fold change | P-value | Name |
|----------------------|-------------|---------|------|
| Pde4a                | −1.20556    | 1.57E-07| phosphodiesterase 4A cAMP specific |
| Eaf2/U19             | −1.23309    | 1.52E-06| ELL associated factor 2, ehrlich S-II transcriptional activator factor testosterone regulated apoptosis inducer and tumor suppressor |
| Sp3                  | −1.24825    | 9.34E-08| trans-acting transcription factor 3 |
| Ndufa4               | −1.26049    | 5.41E-08| NADH dehydrogenase (ubiquinone) 1 alpha subcomplex. assembly factor 4 |
| Wnt11                | −1.28904    | 3.63E-08| wingless-related MMTV integration site 11 |
| Fgfr9p3              | −1.30479    | 5.46E-07| fibroblast growth factor binding protein 3 |
| Ccna1                | −1.31914    | 9.33E-09| cyclin A1 |
| Trim69               | −1.35725    | 5.04E-08| tripartite motif-containing 69 |
| Npy5r                | −1.37768    | 5.33E-10| neuropeptide Y receptor Y5 |
| Zfp78                | −1.38882    | 1.95E-09| zinc finger protein 78 |
| Amhr2                | −1.46255    | 5.42E-09| anti-Mullerian hormone type 2 receptor transforming growth factor beta receptor signalling pathway |
| L3mbt4               | −1.48069    | 4.21E-08| l(3)mbt-like 4 |
| Zscl17               | −1.48573    | 4.10E-08| zinc finger with KRAB and SCAN domains 17 |
| Ngf                  | −1.49018    | 1.63E-08| nerve growth factor |
| Nlrc5                | −1.53839    | 1.64E-08| NLR family. CARD domain containing 5 negative regulation of NF-kappaB transcription factor activity |

The table is highlighting genes selected on the basis of their known involvement in pathways regulating odontogenesis. doi:10.1371/journal.pone.0084343.t009

...and their anatomical definitions. A similar approach was used for molar analysis (Fig. S2).

Calculation involved euclidean distances between selected pairs of points that could be compared to identify statistical significant differences between WT and mutant mice. The methodology is based upon previously described work [63,64]. To avoid any bias in landmarks position, landmarks were placed two times by one investigator (intra-investigator reproducibility) and one time by another investigator (inter-investigator reproducibility).

Figure 8. Summary of experiments involving in vitro inactivation of Rsk2 and its consequences on target gene expression, as observed after microinjection in E14.5 mandibular molar explants of a Rsk2 shRNA, followed by qRT-PCR analysis 24 h after injection. A: Rsk2 was down regulated 4.01 fold after electroporation. B: Eaf2 was significantly down-regulated, and C: Rdh1 was up-regulated after electroporation. Histograms show expression levels in molars electroporated with the random shRNA (blue) and microinjected-electroporated explants with the Rsk2 shRNA (red), with values normalized with respect to Gapdh expression. Data (mean ± SEM) were analyzed with Student t-test; ***p<0.001; **p<0.01; *p<0.05.

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another (inter-investigator reproducibility). As no statistical differences were observed between distances measured within or in between investigators, we decided to base our study on the average of the three measured distances. Statistical analysis comparing WT and mutant mice was performed using Mann-Whitney non-parametric test. P-values lower than 0.05 were considered as significant.

Analysis of incisor morphology

All image processing tasks were done using MicroView (GE Healthcare, Waukesha, USA). Right and left mandibular incisors were isolated by manual segmentation from mandible micro-CT acquisitions of the two mouse groups (Rsk2+/Y and 6 WT mice). Several measurements were achieved for morphological description, on the lateral and dorsal views of a 3D isosurface rendering of incisors (threshold value = 700 Hounsfield units; the length of the longest and external bow (Lj), the height of median part of the incisor (hi), the total height (H), the horizontal length joining the tip of the incisor and the distal extremity of the root (l), projected area in lateral view of the incisor (lpa) and the thickness of the median part of the incisor (t) (Fig. S3). A Wilcoxon-Mann Whitney test was used to compare both groups.

Whole mount in situ hybridization on cryosections

Whole mount in situ hybridizations on mandibles collected at E14.5 was performed with a digoxigenin-labeled Shh riboprobe as described [66], using an Intavis InSituPro robot (for details, see http://www.empress.har.mrc.ac.uk/browser/, Gene Expression section). The Shh template plasmid was kindly provided by A. McMahon (Harvard University).

DNA microarray analysis

Tissue preparation. Embryos at E14.5 were dissected and mandibular molars were isolated. The tail was used for embryo genotyping. Individual tissue samples were frozen in liquid nitrogen and kept at -80°C until genotyping results were available and further used.

Microarray hybridization. To obtain enough RNA for hybridization on DNA microarrays, total RNA was extracted from molar tooth germs dissected manually (pooling samples from two independent wild-type or Rsk2−/− embryos). Total RNA was extracted with the RNAeasy micro Kit (Qiagen). RNA quality was verified by analysis of the 2100 Bioanalyzer (Agilent). All samples displayed a RNA Integrity Number greater than 9.8. Eight Rsk2−/− and 8 WT samples were hybridized and compared.

Biotinylated single strand cDNA targets were prepared, starting from 300 ng of total RNA, using the Ambion WT Expression Kit (Cat #4411974) and the Affymetrix GeneChip WT Terminal Labeling Kit (Cat #900671), according to Affymetrix recommendations. Following fragmentation and end-labeling, 1.5 µg of cDNAs were hybridized for 16 h at 45°C on GeneChip Mouse Gene 1.0 ST arrays (Affymetrix) interrogating 28,853 genes represented by approximately 27 probes spread across the full length of the gene. The chips were washed and stained in the GeneChip Fluidics Station 450 (Affymetrix) and scanned with the GeneChip Scanner 3000 7G (Affymetrix). Finally, raw data (.CEL Intensity files) were extracted from the scanned images using the Affymetrix GeneChip Command Console (AGCC) version 3.1. See also [67].

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) [68] and are accessible through GEO Series accession number GSE51034. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51034)

Microarray analysis. CEL files were further processed with the Partek software to obtain principal component analysis (PCA) and to select and consider only genes with a signal value above 5 (20th percentile of all expression values) in at least one sample. Genes were considered as differentially expressed if the false discovery rate from Benjamini and Hochberg test was under 0.1 and if the fold change was greater than 1.2 or lower than -1.2.

In vitro culture and shRNA inactivation

E14.5 tooth germs were dissected and cultured in chemically defined culture medium as previously described [19]. The tooth explant consisted of both the enamel organ (epithelial component) and the attached dental mesenchyme. A DNA solution containing 1 µg of Rsk2 shRNA (SABiosciences SureSilencing shRNA Plasmid for Mouse Rps6kA3 clone ID1– linearized pGeneclend Neomycin vector, insert sequence TGGATGATCCAGAGGGAATCTCGAGG-AAT) or a random shRNA (insert sequence GGAATCTCAGGATGTGTTGAC, 0.5% sucrose, and Fast Green (Sigma; 1/10,000) was injected into the developing molars with a Femtojet Eppendorf device (t = 0.2 s, P = 61 kPa). Two platinum needle electrodes (0.1 mm tip, Sonidel) were inserted into the tissue laying into a cover round platinum flat electrode (Sonidel). DNA was then transferred into the cells using an ECM 830 Electroporation System (BTX Harvard Apparatus), applying 1 set of 5 pulses. Electroporation settings were set to 5 V for 50 ms, spaced by 50 ms intervals. As negative control, explants were electroporated with a random shRNA sequence.

After electroporation, the explants were embedded into 14 µl rat collagen drops (BD Biosciences) containing 0.2 ng laminin (BD Biosciences), 50 µl DEMEM 10x concentrated, 5 µl HEPES (1 mM), 60 µl 7.5% NaHCO3 (adapted from [24]). The collagen drops were kept on ice to prevent them from polymerizing. The collagen was then polymerized at 37°C for 10–15 min before addition of the culture medium.

All explants were subsequently cultured for 24 hours in a DMEM-HAM F12 medium supplemented with Vitamin C (18 mg/ml); L-Glutamine (200 mM); Streptomycin (1000 U/ ml) and 20% fetal calf serum for optimal tooth growth. Explants were then processed for RT-qPCR analysis.

Real-time quantitative RT-PCR

RT-PCR assays were performed in duplicate, comparing 3 wild-type and 3 Rsk2−/− RNA samples (4 teeth were used in each sample for validation of microarray data), or 6 molar samples electroporated with Rsk2 shRNA and 6 control samples (for shRNA experiments). RNA extractions were performed as previously described (ref. [67]). Oligo-dT primed cDNAs were generated
using the Superscript II kit (Invitrogen) according to the manufac-
turer's protocol. Quantitative real-time PCR was achieved using
SyrbrGreen and LightCycler 480 (Roche). The sequences of primers
used for the various tested genes are given in Table S3 in File S1. A
probe set for detection of mouse Gapdh mRNA (encoded by a
housekeeping gene) was used for normalization. For each sample,
the ratio between signals for the gene of interest and Gapdh was
calculated to normalize concentration values. To verify if genes
were differentially expressed between electroporated and control
samples, the average of ratios were then compared.

Statistical tests
Data, presented as means ± SEM, were compared using Student
t-test (for 2 groups comparison). P-values below 0.05 were considered
as significant.

Supporting Information
Figure S1 Description of the craniofacial anatomical
landmarks used for X-Ray microtomographic analysis
of Rsk mutant mice. (TIF)
Figure S2 Molar landmarks used for the analysis of X-
Ray tomography images. (TIF)
Figure S3 Three-dimensional isosurface rendering of a
right mandibular incisor from a wild-type mouse, in lateral
(A) and dorsal (B) views, depicting the distances measured for
the morphometric analysis. (TIF)

File S1 Supporting Information file containing Tables
S1–S3. Table S1 in file S1. Molar root numbers in WT and
Rsk2−/− mice. UR: upper right. UL: upper left. LR: lower right. LL:
lower left. M1: first molar. M2: second molar. M3: third
molar. ST: supernumerary tooth. Table S2 in file S1. Template
plasmids used for in situ hybridization. Table S3 in file S1. Sequences
of primers used for quantitative RT-PCR experiments.

Author Contributions
Conceived and designed the experiments: VH-L, MP PC AC LV AH PD
AB-Z. Performed the experiments: VL-H MP PM AL TK JD SM FB SP.
Analyzed the data: VL-H LV PD AB-Z. Contributed reagents/materials/
analysis tools: CP AL TK JD MS FB SP. Wrote the paper: VL-H MP LV
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