Keystone genes of mammalian tooth patterning and quantification of their expression

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Abstract

Organ development is regulated by complex interactions of multiple regulatory pathways. These pathways (Wnt, Tgfβ, Fgf, Hh, Eda, Notch) are becoming increasingly better known, with many identified genes having well-characterized effects on the phenotype. We classify genes required for normal organogenesis into different categories that range from essential to subtle modification of the phenotype. We focus on the mouse tooth development in which over 70 genes are known to be required for normal odontogenesis. These genes were classified into progression, shape, and tissue categories based on whether their null mutations cause early developmental arrests, altered morphologies, or hard tissue defects, respectively. Collectively, we call these here the developmental keystone genes. Additionally, we identified 100 developmental genes with no phenotypic effects on molars when null mutated, thereby providing the means to contrast expression dynamics between keystone and non-keystone

genes. Transcriptome profiling using microarray and RNAseq analyses of patterning stage mouse molars show elevated expression levels for progression and shape genes, the former category showing the most significant upregulation. Single-cell RNAseq analyses reveal that even though the size of the expression domain, measured in number of cell, is the main driver of organ-level expression, the progression genes show high cell-level transcript abundances. Furthermore, progression genes, but not shape genes, are upregulated even when compared to the other genes in the pathways into which they belong. The lower upregulation of the shape category is found to be due to the high proportion of locally secreted ligands among the shape genes. We postulate that regulation of shape is highly dependent on spatially diffusing ligands, which are generally more locally expressed compared to their receptors and intracellular components. The combination of phenotypically defined gene categories and organ transcriptomes allow the characterization of the expression dynamics underlying different aspects of organogenesis.

Introduction

Much of the functional evidence for the roles of developmental genes comes from natural mutants or experiments in which the activity of gene is altered. Most often these experiments involve deactivation, or null mutations where the production of a specific gene product is prevented altogether. In the cases where development of an organism is arrested altogether, the specific gene is considered to be absolutely required or essential for development (1, 2). Through a large number of experiments in different organisms, an increasingly nuanced view of developmental regulation has emerged showing that some genes appear to be absolutely required, whereas others may cause milder effects on the phenotype (3, 4). Yet, there are a large number of genes that despite being dynamically regulated during individual organ development, have no detectable phenotypic effect when null mutated.

Within the framework of distinct phenotypic outcomes of gene deactivation, it can be argued for a gradation from developmentally 'more essential' to 'less essential' genes. Collectively, these can be considered to be analogous to the keystone species concept used in ecology (5). These genes, which can be called 'developmental keystone genes', are not necessarily essential for development. Rather, compared to all the genes, developmental keystone genes exert a disproportional effect on the phenotype. To be operational, developmental keystone genes are

defined within an organ (or individual) of interest. This organ focus also means that genes that have no effects in one organ may be critical for the development of another organ. Therefore, in the context of evolution, both the organ specific regulation and protein function would be the targets of natural selection on keystone genes.

As large-scale analyses of transcriptomes result in expression profiles of thousands of genes, it is now possible to address whether there might be any systematic differences between the regulation of keystone and other genes during organogenesis. Here we address such differences using the mammalian tooth. Especially the development of the mouse molar is well characterized, with over 70 genes that are known to be individually required for normal tooth development (6, 7). The detailed effects of null mutations of these genes are also well characterized, ranging from a complete developmental arrest to relatively mild modifications of morphology, or defects in the mineralized hard tissue (6, 7).

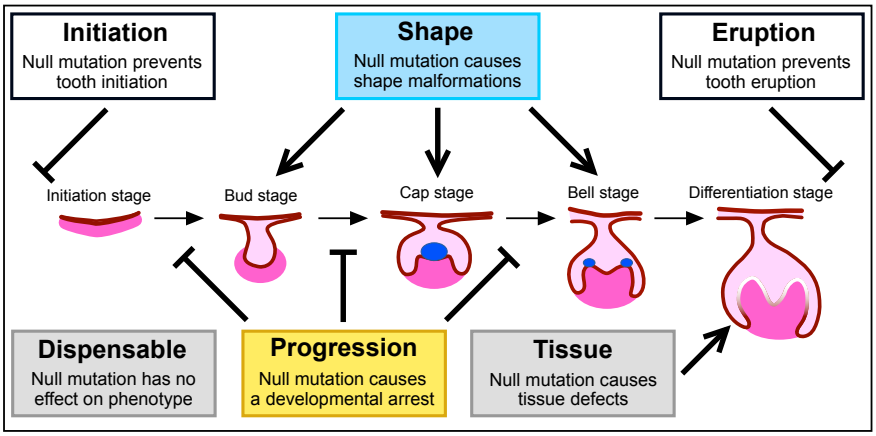

Fig 1. Keystone gene categories of tooth development. Mouse molar development progresses from initiation and patterning to formation of the hard tissues and eruption. These steps are mediated by reciprocal signaling between epithelium (lighter magenta) and mesenchyme (darker magenta). A central step in the patterning is the formation of the epithelial signaling center, the primary enamel knot (dark blue circle inside the cap stage tooth). Several genes are known to be required for the developmental progression and regulation of the shape around the time of cap stage, and here we analyzed transcriptomes in the bud (E13), cap (E14) and bell (E16) stage molars. Expression of progression and shape category genes were compared to tissue and dispensable genes, as also to other developmental process genes. Fever initiation and eruption category genes are known, and they were excluded from the analyses. For listing of the genes, see Appendix S1 and Table S1.

Classification of developmental keystone genes.

Here our main focus is on a critical step in tooth development, namely the formation of the cap stage tooth germ (Fig. 1). At this stage the patterning of tooth crown begins, and the effects of experimental modifications in several signaling pathways first manifest themselves around this time of development (8). To classify the studied genes, we divide them into four different categories. The first category is the **progression category** containing genes that cause a developmental arrest of the tooth when null mutated (Fig. 1, Appendix S1). The second set of genes belongs to the **shape category** and they alter the morphology of the tooth when null mutated. Unlike the progression genes, many of the shape gene caused modifications of teeth are subtle and functional, hence these genes are not strictly essential for tooth development. The third category is the **tissue category** and null mutations in these genes cause defects in the tooth hard tissues, enamel and dentine. Both the progression and shape categories include genes that are required for normal cap-stage formation. In contrast, the tissue category is principally related to the formation of extracellular matrix and these genes are known to be needed later in the development (9). Because there is more than a five-day delay from the cap-stage to matrix secretion in the mouse molar, here we considered the tissue category as a control for the first two categories. Additionally, we compiled a second control set of developmental genes that, while expressed during tooth development, are reported to lack phenotypic effects when null mutated (Table S1). This 'non-keystone' or **dispensable category** is defined purely within our operational framework of identifiable phenotypic effects and we do not imply that these genes are necessarily unimportant even within the context of tooth development. Many genes function in concert and the effects of their deletion only manifests when mutated in combinations (also known as synthetic mutations). We identified five such redundant pairs of paralogous genes and a single gene whose null phenotype surfaces in heterozygous background of its paralogue. Altogether these 11 genes were tabulated separately as a **double category**. In the progression, shape, tissue, and dispensable categories we tabulated 15, 28, 27, and 100 genes respectively (Fig. 1, Appendix S1, Table S1). While still limited, these genes should represent a robust classification of validated experimental effects. We note that these groupings do not exclude the possibility that a progression gene, for example, can also be required for normal hard tissue formation. Therefore, the keystone gene categories can be considered to reflect the temporal order in which they are first required during odontogenesis.

121

122 In addition to the categories studied here, there are genes required for the initiation of tooth
123 development, of which many are also potentially involved in tooth renewal. Because the
124 phenotypic effect of these initiation genes on tooth development precedes the visible
125 morphogenesis, and the phenotype might include complete lack of cells of the odontogenic
126 lineage, we excluded these genes from our analyses. Similarly, we excluded genes preventing
127 tooth eruption with no specific effect on the tooth itself (Appendix S1).

128

129 To examine the keystone genes in the context of whole transcriptomes, we compared the
130 expression levels with all the developmental-process genes (GO:00332502, ref. 10), as also with
131 all the other protein coding genes of the mouse genome.

132

133 **Results**

134 **Progression and shape category genes show elevated expression at the onset of tooth**
135 **patterning**

136 For a robust readout of gene expression profiles, we first obtained gene expression levels using
137 both microarray and RNAseq techniques from E13 (bud stage) and E14 (cap stage) mouse
138 molars (Materials and Methods). From dissected tooth germs we obtained five microarray and
139 seven RNAseq replicates for both developmental stages. The results show that especially the
140 progression category genes are highly expressed during E13 compared to the control gene sets
141 (tissue, dispensable, and developmental-process categories, P values range from 0.0003 to
142 0.0416 for RNAseq and microarray experiments, tested using random resampling, for details
143 and all the tests, see Materials and Methods, Fig. 2, Tables S2, S3). Comparable differences are
144 observed in E14 molars (P values range from 0.0000 to 0.05, Fig. 2, Tables S2, S3).

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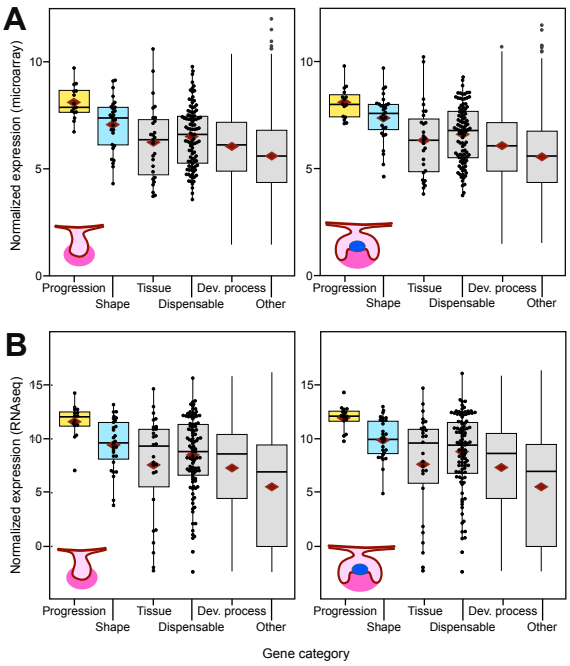
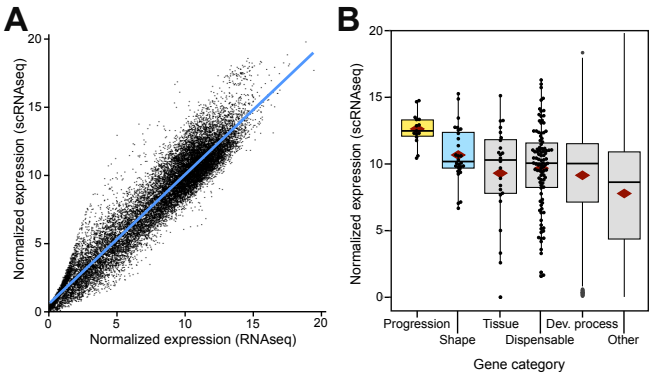


Fig 2. Bud and cap stage mouse molars show elevated expression of keystone genes. (A) Microarray and (B) RNAseq analyses of the transcriptomes of E13 and E14 molars show highly elevated expression of progression category and moderately elevated shape category genes (for tests, see Table S3). Tissue category genes are involved in dentine and enamel formation that begin at birth, around six days from the E14 cap stage. The number of genes having RNAseq expression data in each category are 15, 28, 27, 100, 4106, and 16165 for progression, shape, tissue, dispensable, developmental process, and other, respectively. The corresponding numbers for the microarray data are 15, 28, 27, 98, 3983, and 14825. Boxes enclose 50% of observations; the median and mean are indicated with a horizontal bar and diamond, respectively, and whiskers extend to last values within 1.5 interquartiles. Individual data points are shown for the smaller categories.

In general, the expression differences between progression and tissue categories appear greater than between progression and dispensable categories (P values range from 0.0027 to 0.0416 and 0.0055 to 0.05, respectively, Table S3), suggesting that some of the genes in the dispensable category may still play a functional role in tooth development. In our data we have 11 genes that cause a developmental arrest of the tooth when double mutated (Appendix S1). The expression level of this double-mutant category shows incipient upregulation compared to that of the developmental-process category (P values range from 0.0322 to 0.1796 Table S3), but not when compared to the tissue or dispensable categories (P values range from 0.0931 to 0.5007, Table S3). Therefore, it is plausible, based on the comparable expression levels between double and

167 dispensable categories, that many of the genes in the dispensable category may cause
168 phenotypic effects when mutated in pairs.
169
170 Even though the shape category expression levels are lower than that of the progression
171 category (Fig. 2), at least the E14 microarray data suggests elevated expression levels relative to
172 all the other control categories (P values range from 0.0002 to 0.0383, Table S3). The
173 moderately elevated levels of expression by the shape category genes could indicate that they
174 are required slightly later in development, or that the most robust upregulation happens for
175 genes that are critical for the progression of the development. The latter option seems to be
176 supported by a RNAseq analysis of E16 molar, showing only slight upregulation of shape
177 category genes in the bell stage (Table S3).
178
179 One complication of our expression level analyses is that these have been done at the whole
180 organ level. Because many of the genes regulating tooth development are known to have
181 spatially complex expression patterns within the tooth, cell-level examinations are required to
182 decompose the patterns within the tissue.
183
184 **Single-cell RNAseq reveals cell-level patterns of keystone genes**
185 Tooth development is punctuated by iteratively forming epithelial signaling centers, the enamel
186 knots. The first, primary enamel knot, is active in E14 molar and at this stage many genes are
187 known to have complex expression patterns. Some progression category genes have been
188 reported to be expressed in the enamel knot, whereas others have mesenchymal or complex
189 combinatorial expression patterns (8, 9). To quantify these expression levels at the cell-level, we
190 performed a single-cell RNAseq (scRNAseq) on E14 molars (Material and Methods). We
191 focused on capturing a representative sample of cells by dissociating the tooth germs without
192 cell sorting (N = 4). After data filtering, 7000 to 8811 cells per tooth were retained for the
193 analyses, providing 30930 aggregated cells for a relatively good proxy of the E14 tooth
194 (Material and Methods).
195
196



197

198 **Fig 3. Single cell RNAseq data reflect the bulkRNA analyses.** (A) Bulk and scRNAseq expression
199 levels show overall correspondence in tissue level expression in E14 molar. (B) Progression category
200 genes show the strongest upregulation whereas shape category is intermediate between the progression
201 and other categories (for tests, see Table S3). Boxes in (B) enclose 50% of observations; the median and
202 mean are indicated with a horizontal bar and diamond, respectively, and whiskers extend to last values
203 within 1.5 interquartiles. Individual data points are shown for the smaller categories.

204

205 First we examined whether the scRNAseq produces comparable expression levels to our
206 previous analyses. For the comparisons, the gene count values from the cells were summed up
207 and treated as bulk RNAseq data (Fig. 3A, Material and Methods). We analyzed the expression
208 levels of different gene categories as in the bulk data (Fig 2) and the results show a general
209 agreement between the experiments (Fig. 2, 3B). As in the previous analyses (Table S3), the
210 progression category shows the highest expression levels compared to the control gene sets (P
211 values range from 0.0076 to 0.0309, Table S3). Although the mean expression of the shape
212 category is intermediate between progression and control gene sets, scRNAseq shape category
213 is not significantly upregulated in the randomization tests (P values range from 0.7825 to
214 0.9971). This pattern reflects the bulk RNAseq analyses while the microarray data showed
215 stronger upregulation (Fig. 2), suggesting a potentially discernable but subtle upregulation of
216 shape category genes.

217

218 Unlike the bulk transcriptome data, the scRNAseq data can be used to quantify the effect of size
219 expression domain size. The importance of expression domain size is well evident in the
220 scRNAseq data when we calculated the number of cells that express each gene (Material and
221 Methods). The data shows that the overall tissue level gene expression is highly correlated with
222 the cell population size (Fig. 4A). In other words, the size of the expression domain is the key

driver of expression levels measured at the whole tissue level.

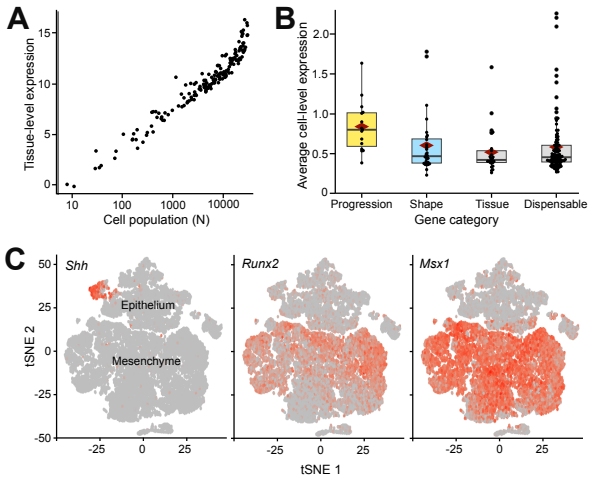


Fig 4. Expression domain size and high cell-level transcript abundance of progression genes. (A) The size of the expression domain, measured as the number of cells, is the key driver of expression levels measured at the whole tissue level. (B) Progression category shows high cell-level expression or transcript abundance, indicating high expression relative to the expression domain size The P values for progression, shape, and tissue categories compared to dispensable category are 0.0008, 0.7009, 0.3413, respectively (one-tailed significance levels obtained using 10,000 permutations). (C) tSNE plots showing diverse expression patterns of three different progression category genes in the scRNAseq data. Boxes in (B) enclose 50% of observations; the median and mean are indicated with a horizontal bar and diamond, respectively, and whiskers extend to last values within 1.5 interquartiles.

To examine the cell level patterns further, we calculated the mean transcript abundances for each gene for the cells that express that gene (see Material and Methods). This metric approximates the cell-level upregulation of a particular gene, and is thus independent of the size of the expression domain. We calculated the transcript abundance values for each gene in each cell that expresses progression, shape, tissue, double, and dispensable gene categories. The resulting mean transcript abundances were contrasted to that of the dispensable category (Material and Methods). The results show that the average transcript abundance is high in the progression category whereas the other categories show roughly comparable transcript abundances (Fig. 4B). Considering that the progression category genes have highly heterogeneous expression patterns (e.g., Fig. 4C), their high cell-level transcript abundance (Fig. 4B) is suggestive about the critical role of these genes at the cell level. That is, progression

247 category genes are not only highly expressed at the tissue level because they have broad
248 expression domains, but rather because they are upregulated in individual cells irrespective of
249 the domain size. These results suggest that high cell-level transcript abundance is a
250 characteristic feature of keystone genes required for the progression of odontogenesis. We note
251 that although the dispensable category has several genes showing comparable expression levels
252 with that of the progression category genes at the tissue level (Fig. 2), their cell-level transcript
253 abundances are predominantly low (Fig. 4B).
254
255 Next we examined more closely the differences between progression and shape category genes,
256 and to what extent the upregulation of the keystone genes reflect the overall expression of the
257 corresponding pathways.
258
259 **Keystone gene upregulation in the context of their pathways**
260 In our data the developmental-process genes appear to have slightly elevated expression levels
261 compared to the other protein coding genes (Figs 2, 3B), suggesting an expected and general
262 recruitment of the pathways required for organogenesis. To place the progression and shape
263 category genes into the specific context of their corresponding pathways, we investigated
264 whether the pathways implicated in tooth development show elevated expression levels. Six
265 pathways, FGF, Wnt, Tgfβ, Hedgehog (Hh), Notch, and Ectodysplasin (Eda), contain the
266 majority of progression and shape genes (Materials and Methods). First we used the RNAseq of
267 E14 stage molars to test whether these pathways show elevated expression levels. We manually
268 identified 272 genes belonging to the six pathways (Materials and Methods, Table S4).
269 Comparison of the median expression levels of the six-pathway genes with the developmental-
270 process genes shows that the pathway genes are a highly upregulated set of genes (Fig. 5A, $P <$
271 0.0001, random resampling). This difference suggests that the experimentally identified
272 progression and shape genes might be highly expressed partly because they belong to the
273 developmentally upregulated pathways. To specifically test this possibility, we contrasted the
274 expression levels of the progression and shape genes to the genes of their corresponding
275 signaling families.
276

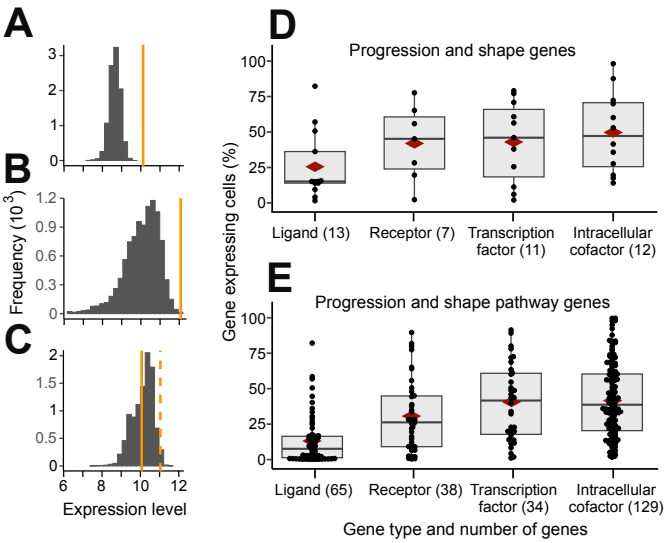


Fig 5. Restricted expression of ligands explains differences in upregulation. (A) Compared to all the developmental process genes, the pathways containing progression and shape category genes are generally upregulated ($P < 0.0001$, orange line, random sampling using median expression levels and 10,000 permutations). (B) Median expression of progression genes (orange line) shows further upregulation compared to all the genes in the corresponding pathways ($P = 0.0004$). (C) Median expression of shape category genes (orange line) shows comparable expression with the genes in the corresponding pathways ($P = 0.5919$), but excluding ligands makes the shape category genes highly expressed ($P = 0.0154$, orange dashed line). (D) Of the progression and shape category genes, ligands are expressed in fewer number of cells compared to the non-secreted genes. (E) Ligands are also expressed in relatively few cells among all the pathway genes. The P value for ligands in (D) and (E) are $P < 0.0001$ (random sampling compared to all the other gene types). Boxes in (D, E) enclose 50% of observations; the median and mean are indicated with a horizontal bar and diamond, respectively, and whiskers extend to last values within 1.5 interquartiles.

The 15 progression category genes belong to four signaling families (Wnt, Tgfβ, Fgf, Hh) with 221 genes in our tabulations. Even though these pathways are generally upregulated in the E14 tooth, the median expression level of the progression category is still further elevated (Fig. 5B, $P < 0.0001$). In contrast, the analyses for the 28 shape category genes and their corresponding pathways (272 genes from Wnt, Tgfβ, Fgf, Hh, Eda, Notch) show comparable expression levels (Fig. 5C, $P = 0.5919$). Whereas this contrasting pattern between progression and shape genes within their pathways may explain the subtle upregulation of the shape category (Fig. 2), the difference warrants a closer look. Examination of the two gene categories reveals that compared to the progression category genes, relatively large proportion of the shape category genes are

ligands (36% shape genes compared to 20% progression genes, Appendix S1). In our scRNAseq data, ligands show generally smaller expression domains than other genes (roughly by half, Fig. 5D, E), and the low expression of the shape category genes seems to be largely driven by the ligands (Fig. 5C, Table S5).

Overall, the upregulation of the keystone genes within their pathways appears to be influenced by the kind of proteins they encode. In this context it is noteworthy that patterning of tooth shape requires spatial regulation of secondary enamel knots and cusps, providing a plausible explanation for the high proportion of genes encoding diffusing ligands in the shape category.

Discussion

Identification and mechanistic characterization of developmentally essential or important genes has motivated a considerable research effort (e.g. 1–4, 6). One general realization has been that despite the large number of genes being dynamically expressed during organogenesis, only a subset appears to have discernable effects on the phenotype. This parallels with the keystone species concept used in ecological research (5). Keystone species, that may include relatively few species in a community, are thought to have disproportionally large influence on their environment. Similarly, developmental keystone genes have disproportionally large effects on the phenotypic outcome of their system. Here we considered keystone genes strictly within a developmental system, and these genes, or more accurately their protein products, can be understood as keystone resources (11) that are required for normal development. We note that whereas keystone genes can also be considered within the context of their effects on ecosystems (12), here we limit the explanatory level to a specific organ system. In our case, the 'ecosystem' has been a developing mammalian tooth.

We took advantage of the in-depth knowledge on the details of the phenotypic effects of various developmental genes (Appendix S1). This allowed us to classify genes into different categories that reflect their functional role during organogenesis. Furthermore, a multitude of studies have made it possible to identify a dispensable or non-keystone gene category. Obviously, as in ecological data, our category groupings can be considered a work in progress as new genes and reclassifications are bound to refine the patterns. Nevertheless, our analyses should provide some robust inferences.

333

334 Most notably, genes that are individually required for the progression of mouse molar
335 development were found to be highly expressed (Figs 2, 3). These genes were highly expressed
336 even within their pathways (Fig. 5A,B) and had markedly high cell-level transcript abundances
337 (Fig. 4B). The high expression level of these progression category genes may well signify their
338 absolute requirement during the cap stage of tooth development. Indeed, it is typically by this
339 stage that a developmental arrest happens when many of the progression genes are null mutated.
340 Interestingly, mice heterozygous for the null-mutated progression genes appear to have normal
341 teeth (Appendix S1). A possible hypothesis to be explored is to examine whether the high cell-
342 level transcript abundance of the progression category is a form of haplosufficiency in which the
343 developmental system is buffered against mutations affecting one allele. Another possibility,
344 that has some experimental support (13), is that there are regulatory feedbacks to boost gene
345 expression to compensate for a null-allele. In contrast to the progression category, gene pairs
346 arresting tooth development as double mutants have relatively low expression levels, perhaps
347 suggestive that many genes in the dispensable category could be redundant to each other.

348

349 Evolutionarily, because all the studied progression and shape category genes are involved in the
350 development of multiple organ systems, our results may point to cis-regulatory differences that
351 specifically promote the expression of these genes in an organ specific manner. Consequently,
352 species that are less reliant on teeth (e.g., some seals) or have rudimentary teeth (e.g., baleen
353 whales) can be predicted to have lowered expression levels of the progression genes. At an
354 organism level, our gene categories should not be considered as indicative of having simple
355 effects on individual fitness. For example, in our specific case of teeth, defects on enamel may
356 be more costly for the mammalian parent than, for example, a dominant null mutation causing
357 an arrest of tooth development with comparable defects on other organ systems.

358

359 Considering the numerous genes expressed in a developing organ system, our results point to
360 the potential to use cell-level expression levels to identify other genes critical for organogenesis.
361 Here the single-cell transcriptomes provided a more nuanced view into the spatial patterns of the
362 different gene categories than the tissue level transcriptomes alone. In our tabulation over a third
363 of the shape category genes were ligands. Tooth shape patterning involves spatial placement of
364 signaling centers that in turn direct the growth and folding of the tissue (8). The involvement of

365 several secreted ligands in this patterning process, and consequently in the shape category, is
366 likely to reflect the requirement of the developmental machinery to produce functional cusp
367 patterns. These cusp patterns are also a major target of natural selection because evolutionary
368 diversity of mammalian teeth is largely made from different configurations of cusps. At the
369 same time, partly due to ligands having generally more restricted expression domains compared
370 to receptors and intracellular proteins, the shape category expression levels were found to be
371 generally lower than that of the progression category. That ligands tend to have smaller
372 expression domains whereas receptors have broader expression domains for tissue competence
373 has been recognized in many individual studies (e.g. refs 14 and 15, and partly in 16), but our
374 analyses suggest that this is a general principle detectable in large-scale transcriptome data. This
375 pattern is also compatible with the classic concepts of tissue competence and evocators or
376 signals produced by organizers (17). Nevertheless, it remains to be explored how the low signal-
377 competence ratio emerges from highly heterogeneous expression domains of various genes. In
378 our data, at least the ligand *Eda* is expressed in a larger number of cells than its receptor *Edar*,
379 suggesting that there are individual exceptions to the general pattern. Another potentially
380 interesting observation is that *Sostdc1* and *Fst*, both secreted sequesters or inhibitors of
381 signaling, were among the most broadly expressed of the ligands. Thus, at least some of the
382 exceptions to the low signal-competence ratio may be modulators of tissue competence.
383
384 In conclusion, genes critical for tooth patterning are highly expressed, but the level of
385 upregulation is influenced by the kind of proteins they encode. Combining phenotypic
386 classifications of the roles of genes in development with transcriptomes enables new ways to
387 integrate experimental data with development. With advances in the analyses of transcriptomes
388 and gene regulation, it will be possible to explore experimental data from other organs and
389 species to test and identify system level principles of organogenesis.
390

391 **Material and Methods**

392 **Classification of genes.** We performed extensive literature review of genes that are expressed in
393 developing tooth. The genes were divided into categories based on the effect that their null
394 mutation has on the development of the first mandibular molar in the mouse. Full null-mutant
395 mouse information was used whenever available. Because many developmental genes function
396 in multiple organs and stages during development, full null mutants of several genes are lethal

397 before tooth development even begins. Therefore, we also used information based on the tooth
398 phenotypes of conditional mutant mice (in eight cases, Appendix S1). The effect of conditional
399 mutants can be milder and in our data we have four shape genes that could potentially be in the
400 progression category. To test for overall the robustness of the patterns, we analyzed the
401 expression levels also after combining the progression and shape category genes, and the pattern
402 of overall upregulation remained largely the same.
403
404 Genes that are individually indispensable for normal tooth development were classified as
405 keystone genes. Conversely, genes, which loss-of-function mutation has no effect on tooth
406 development are non-keystone genes. The tabulation of these dispensable genes was done from
407 published reports and by inspecting published figures and data. Keystone genes were further
408 divided into four categories based on the type of effect their loss of function has on tooth
409 development. The progression category is defined by a null mutant phenotype that is a
410 developmental arrest of the tooth. The shape category has the genes whose null mutations alter
411 the morphology (or shape) of the tooth. The tissue category contains the genes whose null
412 mutations cause defects in the tooth hard tissues. Only the progression genes can be considered
413 essential for organ development per se, whereas the effects of many of the shape category genes
414 can be quite subtle.
415
416 We created a manually curated list of genes in the six key pathways (Wnt, TgfB, Fgf, Hh, Eda,
417 Notch) based on review publications and allocated the keystone genes into these pathways
418 where appropriate. The keystone genes, non-keystone genes, and the pathway genes were also
419 classified as 'ligand (signal), 'receptor', 'intracellular molecule', 'transcription factor' or
420 'other'. Because these kinds of classifications are not always trivial as some biological
421 molecules have multiple functions in the cell, we used the inferred primary role in teeth. The
422 developmental-process genes with GO term "GO:0032502" and experimental evidence codes
423 were obtained from R package "org.Mm.eg.db" (18). Only curated RefSeq genes are used in
424 this study. All tabulations are in Appendix S1 and Table S1, and S4.
425
426 **Dissection of teeth.** Wild type tooth germs were dissected from embryonic stages
427 corresponding to E13, E14 and E16 molar development. For bulk and single-cell RNAseq we
428 used C57BL/6JOlaHsd mice, and for microarray we used NMRI mice. Minimal amount of

429 surrounding tissue was left around the tooth germ, at the same time making sure that the tooth
430 was not damaged in the process. The tissue was immediately stored in RNAlater (Qiagen
431 GmbH, Hilden, Germany) for RNAseq or in TRI Reagent (Merck, Darmstadt, Germany) in -
432 80°C for microarray. Each tooth was handled individually. Seven biological replicates were
433 made for RNAseq and five for microarray. Numbers of left and right teeth were balanced.
434
435 **RNA extraction.** The tooth germ was homogenised into TRI Reagent (Merck, Darmstadt,
436 Germany) using Precellys 24 -homogenizer (Bertin Instruments, Montigny-le-Bretonneux,
437 France). The RNA was extracted by guanidium thiocyanate-phenol-chloroform method and then
438 further purified by RNeasy Plus micro kit (Qiagen GmbH, Hilden, Germany) according to
439 manufacturer's instructions. The RNA quality was assessed for some samples with 2100
440 Bioanalyzer (Agilent, Santa Clara, CA) and all the RIN values were above 9. The purity of
441 RNA was analysed by Nanodrop microvolume spectrophotometer (ThermoFisher Scientific,
442 Waltham, USA). RNA concentration was measured by Qubit 3.0 Fluorometer (ThermoFisher
443 Scientific, Waltham, USA). The cDNA libraries were prepared with Ovation Universal RNAseq
444 System (Tecan, Zürich, Schwitzerland).
445
446 **Bulk RNA sequencing.** Gene expression levels were measured both in microarray (Affymetrix
447 Mouse Exon Array 1.0, GPL6096) and RNAseq (platforms GPL19057, Illumina NextSeq 500).
448 The microarray gene signals were normalized with aroma.affymetrix (19) package using
449 Brainarray custom CDF (Version 19, released on Nov 13, 2014) (20). Whereas the RNAseq
450 reads (84 bp) were evaluated and bad reads are filtered out using FastQC (21), AfterQC (22)
451 and Trimmomatic (23). This has resulted into on average 63 millions reads per sample. Then
452 good reads were aligned with STAR (24) to (GRCm38/mm10/Ensembl release 90 - August
453 2017) and counts for each gene was performed by HTSeq (25) tool. On average 85% of reads
454 were uniquely mapped to the genome. The sequencing data is available in GEO under the
455 accession number XXXX.
456
457 **Single cell RNA sequencing.** Each tooth was processed individually in the single-cell
458 dissociation. The tooth germ was treated with 0.1 mg/ml liberase (Roche, Basel, Schwitzerland)
459 in Dulbecco's solution for 15 min at 28°C in shaking at 300 rpm followed by gentle pipetting to
460 detach the mesenchymal cells. Then the tissue preparation was treated with TrypLE Select (Life

461 Technologies, Waltham, USA) for 15 min at 28°C in shaking at 300 rpm followed by gentle
462 pipetting to detach the epithelial cells. The cells were washed once in PBS with 0,04% BSA.
463 The cells were resuspended in 50 µl PBS with 0.04% BSA. We used the Chromium single cell
464 3' library & gel bead Kit v3 (10x Genomics, Pleasanton, USA). In short, all samples and
465 reagents were prepared and loaded into the chip. Then, Chromium controller was used for
466 droplet generation. Reverse transcription was conducted in the droplets. cDNA was recovered
467 through emulsification and bead purification. Pre-amplified cDNA was further subjected to
468 library preparation. Libraries were sequenced on an Illumina Novaseq 6000 (Illumina, San
469 Diego, USA).
470
471 **Data analysis.** For scRNAseq, 10x Genomics Cell Ranger v3.0.1 pipelines were used for data
472 processing and analysis. The "cellranger mkfastq" was used to produce fastq files and
473 "cellranger count" to perform alignment, filtering and UMI counting. Alignment was done
474 against mouse genome GRCm38/mm10. The resultant individual count data were finally
475 aggregated with "cellranger aggr". Further, the filtered aggregated feature-barcode matrix was
476 checked for quality and normalization using R package Seurat (26). Only cells with ≥ 20 genes
477 and genes expressed in at least 3 cells were considered for all the downstream analysis. For a
478 robust set of cells for the expression level calculations, we limited the analyses to 30930 cells
479 that had transcripts from 3000 to 9000 genes (7000 to 180000 unique molecular identifiers) with
480 less than 10% of the transcripts being mitochondrial. For comparison with bulk RNAseq data
481 (Figs 3), single-cell data was normalized with DeSeq2 (27) together with the corresponding bulk
482 RNAseq samples, and median expression levels were plotted. The average cell-level expression
483 (Fig. 4B) of a gene *X* was calculated as

484 $$X = \frac{\sum_{k=1}^{n} NX_k}{\sum[NX_k > 1]} ,$$

485 where NX_k is normalized expression of gene *X* in cell *k* and the denominator is the count of cells
486 with non-zero reads. All statistical tests corresponding to Tables S3 and S4 were performed
487 using R package "rcompanion" (28) and custom R scripts.
488

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