another color that activates an approach motivation) would be more beneficial.

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9. We recognize that red and blue can also imply other associations. For example, red might connote excitement or femininity, whereas blue might suggest sadness. Although we acknowledge these different associations, we simply suggest that in the cognitive task domain, red is predominately associated with dangers and mistakes and blue with openness and peace. In addition, we note that the same color may have different associations across cultures (*28*). All our studies were run in a North American university. Thus, future research should test whether our results can be generalized to other cultures.
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Supporting Online Material

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Figs. S1 to S3
Tables S1 to S3
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Self-Sustained Replication of an RNA Enzyme

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An RNA enzyme that catalyzes the RNA-templated joining of RNA was converted to a format whereby two enzymes catalyze each other's synthesis from a total of four oligonucleotide substrates. These cross-replicating RNA enzymes undergo self-sustained exponential amplification in the absence of proteins or other biological materials. Amplification occurs with a doubling time of about 1 hour and can be continued indefinitely. Populations of various cross-replicating enzymes were constructed and allowed to compete for a common pool of substrates, during which recombinant replicators arose and grew to dominate the population. These replicating RNA enzymes can serve as an experimental model of a genetic system. Many such model systems could be constructed, allowing different selective outcomes to be related to the underlying properties of the genetic system.

A long-standing research goal has been to devise a nonbiological system that undergoes replication in a self-sustained manner, brought about by enzymatic machinery that is part of the system being replicated. One way to realize this goal, inspired by the notion of primitive RNA-based life, would be for an RNA enzyme to catalyze the replication of RNA molecules, including the RNA enzyme itself (*1*–*4*). This has now been achieved in a cross-catalytic system involving two RNA enzymes that catalyze each other's synthesis from a total of four component substrates.

The "R3C" RNA enzyme is an RNA ligase that binds two oligonucleotide substrates through Watson-Crick pairing and catalyzes nucleophilic attack of the 3′-hydroxyl of one substrate on the 5′-triphosphate of the other, forming a 3′,5′-phosphodiester and releasing inorganic pyrophosphate (*5*). The R3C ligase was configured to self-replicate by joining two RNA molecules to produce another copy of itself (*6*). This process was inefficient because the substrates formed a nonproductive complex that limited the extent of exponential growth, with a doubling time of about 17 hours and no more than two successive doublings.

The R3C ligase subsequently was converted to a cross-catalytic format (Fig. 1A), whereby a plus-strand RNA enzyme (E) catalyzes the joining of two substrates (A′ and B′) to form a minus-strand enzyme (E′), which in turn catalyzes the joining of two substrates (A and B) to form a new plus-strand enzyme (*7*, *8*). This too was inefficient because of the formation of nonproductive complexes and the slow underlying rate of the two enzymes. The enzymes E and E′ operate with a rate constant of only ~0.03 min^{-1} and a maximum extent of only 10 to 20% (*9*). These rates are about 10 times slower than that of the parental R3C ligase (*5*), and when the two cross-catalytic reactions are carried out within a common mixture the rates are even slower (*7*).

The catalytic properties of the cross-replicating RNA enzymes were improved by the use of in vitro evolution, optimizing the two component reactions in parallel and seeking solutions that would apply to both reactions when conducted in the cross-catalytic format (*9*). The 5′-triphosphate–bearing substrate was joined to the enzyme via a hairpin loop (B′ to E and B to E′), and nucleotides within both the enzyme and the separate 3′-hydroxyl–bearing substrate (A′ and A) were randomized at a frequency of 12% per position. The two resulting populations of molecules were subjected to six rounds of stringent in vitro selection, selecting for their ability to react in progressively shorter times, ranging from 2 hours to 10 ms. Mutagenic polymerase chain reaction was performed after the third round to maintain diversity in the population. After the sixth round, individuals were cloned from both populations and sequenced. There was substantial sequence variability among the clones, but all contained mutations just upstream from the ligation junction that resulted in a G•U wobble pair at this position.

The G•U pair was installed in both enzymes and both 3′-hydroxyl–bearing substrates (Fig. 1B). In the trimolecular reaction (with two separate substrates), the optimized enzymes E and E′ exhibited a rate constant of 1.3 and 0.3 min^{-1} with a maximum extent of 92 and 88%, respectively. The optimized enzymes underwent robust exponential amplification at a constant temperature of 42°C, with more than 25-fold amplification after 5 hours, followed by a leveling off as the supply of substrates became depleted (Fig. 2A). The data fit well to the logistic growth equation $[E]_t = a/(1 + be^{-ct})$, where $[E]_t$ is the concentration of E (or E′) at time t, a is the maximum extent of growth, b is the degree of sigmoidicity, and c is the exponential growth

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rate. For the enzymes E and E′, the exponential growth rate was 0.92 and 1.05 hour^{-1}, respectively.

Exponential growth can be continued indefinitely in a serial transfer experiment in which a portion of a completed reaction mixture is transferred to a new reaction vessel that contains a fresh supply of substrates. Six successive reactions were carried out in this fashion, each 5 hours in duration and transferring 4% of the material from one reaction mixture to the next. The first mixture contained 0.1 μM E and 0.1 μM E′, but all subsequent mixtures contained only those enzymes that were carried over in the transfer. Exponential growth was maintained throughout 30 hours total of incubation, with an overall amplification of greater than 10^8-fold for each of the two enzymes (Fig. 2B).

It is possible to construct variants of the cross-replicating RNA enzymes that differ in the regions of Watson-Crick pairing between the cross-catalytic partners without markedly affecting replication efficiency. These regions are located at the 5′ and 3′ ends of the enzyme (Fig. 1B). Four nucleotide positions at both the 5′ and 3′ ends were varied, adopting the rule that each region contains one G•C and three A•U pairs so that there would be no substantial differences in base-pairing stability. Of the 32 possible pairs of complementary sequences for each region, 12 were chosen as a set of designated pairings (Fig. 1C). Each pairing was associated with a particular sequence within the catalytic core of both members of a cross-replicating pair. Twelve pairs of cross-replicating enzymes were synthesized, as well as the 48 substrates (12 each of A, A′, B, and B′) necessary to support their exponential amplification. Each replicator was individually tested and demonstrated varying levels of catalytic activity and varying rates of exponential growth (fig. S1). The pair shown in Fig. 1B (now designated E1 and E1′) had the fastest rate of exponential growth, achieving about 20-fold amplification after 5 hours. The various cross-replicating enzymes shown in Fig. 1C had the following rank order of replication efficiency: E1, E10, E5, E4, E6, E3, E12, E7, E9, E8, E2, E11. The top five replicators all achieved more than 10-fold amplification after 5 hours, and all except E11 achieved at least fivefold amplification after 5 hours.

A serial-transfer experiment was initiated with a 0.1 μM concentration each of E1 to E4 and E1′ to E4′ and a 5.0 μM concentration of each of the 16 corresponding substrates. Sixteen successive transfers were carried out over 70 hours, transferring 5% of the material from one reaction mixture to the

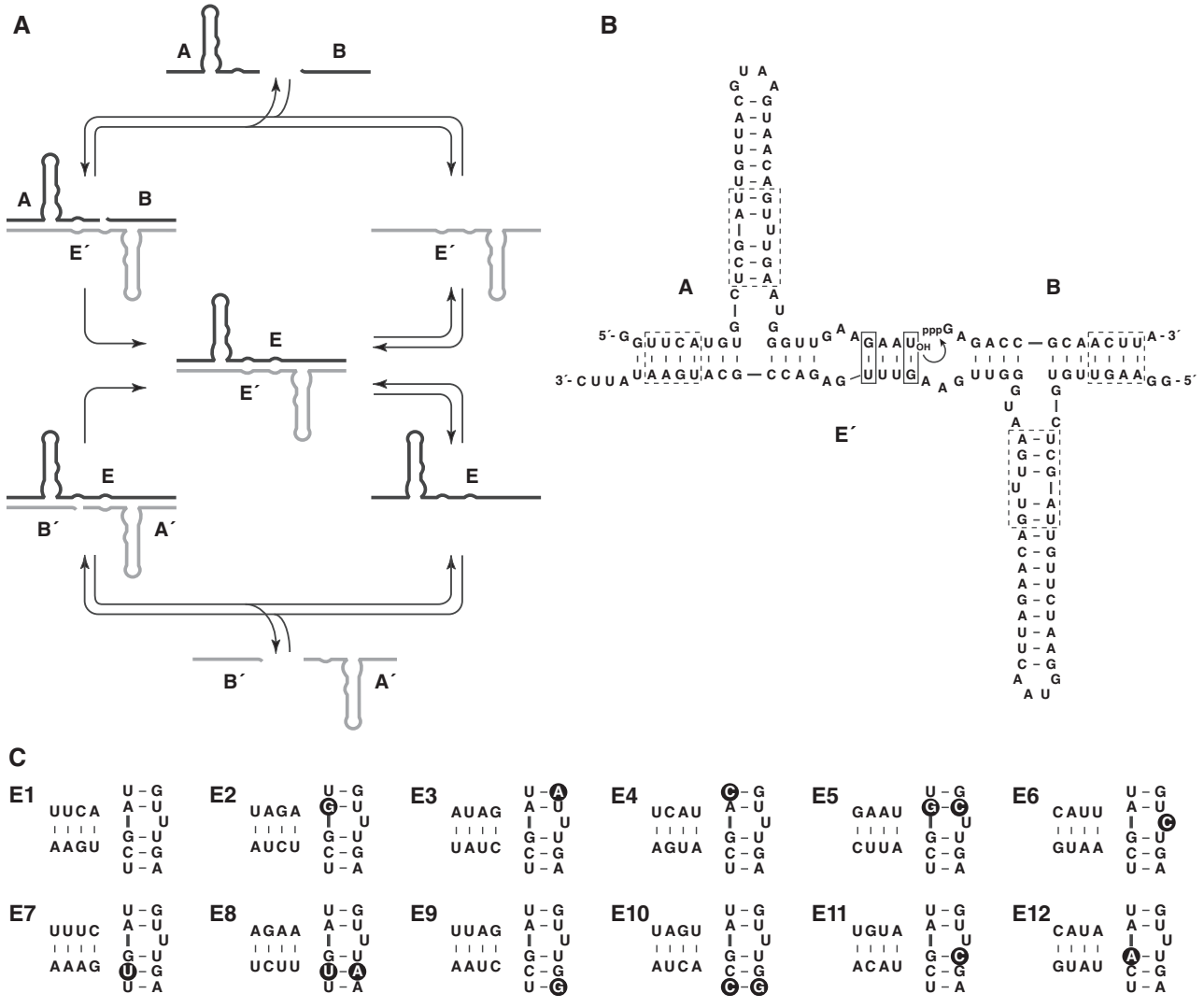

Fig. 1. Cross-replicating RNA enzymes. (**A**) The enzyme E′ (gray) catalyzes ligation of substrates A and B (black) to form the enzyme E, whereas E catalyzes ligation of A′ and B′ to form E′. The two enzymes dissociate to provide copies that can catalyze another reaction. (**B**) Sequence and secondary structure of the complex formed between the enzyme and its two substrates (E′, A, and B are shown; E, A′, and B′ are the reciprocal). The curved arrow indicates the site of ligation. Solid boxes indicate critical wobble pairs that provide enhanced catalytic activity. Dashed boxes indicate paired regions and catalytic nucleotides that were altered to construct various cross replicators. (**C**) Variable portion of 12 different E enzymes. The corresponding E′ enzymes have a complementary sequence in the paired region and the same sequence of catalytic nucleotides (alterations relative to the E1 enzyme are highlighted).

next (fig. S2A). Individuals were cloned from the population after the final reaction and sequenced. Among 25 clones (sequencing E′ only), there was no dominant replicator (fig. S2B). E1′, E2′, E3′, and E4′ all were represented, as well as 17 clones that were the result of recombination between a particular A′ substrate and one of the three B′ substrates other than its original partner (or similarly for A and B). Recombination occurs when an enzyme binds and ligates a mismatched substrate. In principle, any A could become joined to any B or B′, and any A′ could become joined to any B′ or B, resulting in 64 possible enzymes. The set of replicators were designed so that cognate substrates have a binding advantage of several kilocalories per mole as compared with noncognate substrates (fig. S2C), but once a mismatched substrate is bound and ligated, it forms a recombinant enzyme that also can cross-replicate. Recombinants can give rise to other recombinants, as well as revert back to nonrecombinants. Based on relative binding affinities, there are expected to be preferred pathways for mutation, primarily involving substitution among certain A′ or among certain B components (fig. S2D).

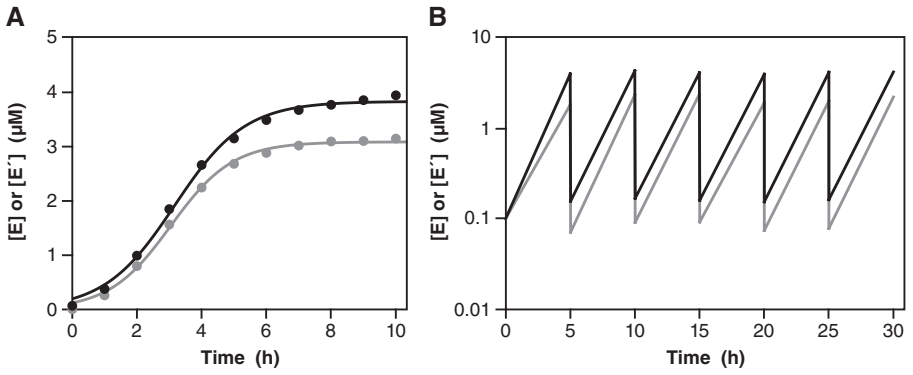

Fig. 2. Self-sustained amplification of cross-replicating RNA enzymes. (**A**) The yield of both E (black curve) and E′ (gray curve) increased exponentially before leveling off as the supply of substrates became exhausted. (**B**) Amplification was sustained by performance of a serial transfer experiment, allowing approximately 25-fold amplification before transferring 4% of the mixture to a new reaction vessel that contained a fresh supply of substrates. The concentrations of E and E′ were measured at the end of each incubation.

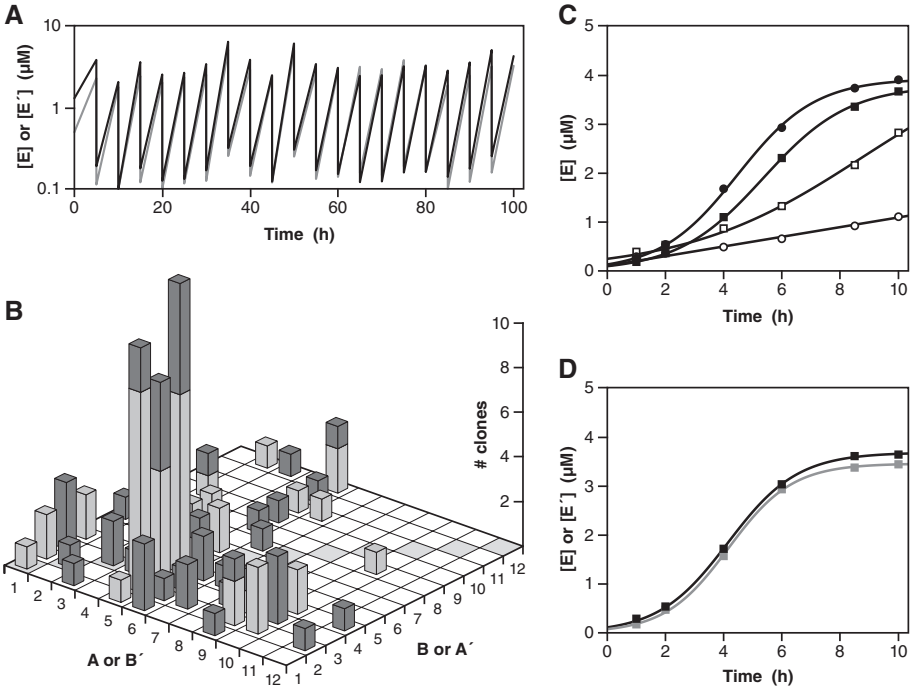

Fig. 3. Self-sustained amplification of a population of cross-replicating RNA enzymes, resulting in selection of the fittest replicators. (**A**) Beginning with 12 pairs of cross-replicating RNA enzymes (Fig. 1C), amplification was sustained for 20 successive rounds of ~20-fold amplification and 20-fold dilution. The concentrations of all E (black) and E′ (gray) molecules were measured after each incubation. (**B**) Graphical representation of 50 E and 50 E′ clones (dark and light columns, respectively) that were sequenced after the last incubation. The A and B (or B′ and A′) components of the various enzymes are shown on the horizontal axes, with nonrecombinant enzymes indicated by shaded boxes along the diagonal. The number of clones containing each combination of components is shown on the vertical axis. (**C**) Comparative growth of E1 (circles) and A5B3 (squares) in the presence of either their cognate substrates alone (solid symbols) or all substrates that were present during serial transfer (open symbols). (**D**) Growth of A5B3 (black curve) and B5′A3′ (gray curve) in the presence of the eight substrates (A5, B2, B3, B4, B5′, A2′, A3′, and A4′) that comprise the three most abundant cross-replicating enzymes.

In the presence of their cognate substrates alone, E1 remained the most efficient replicator, but in the presence of all 48 substrates, the most efficient replicator was A5B3 (Fig. 3C). When the A5B3 replicator was provided with a mixture of substrates corresponding to the components of the three most abundant recombinants, its exponential growth rate was the highest measured for any replicator (Fig. 3D). The fitness of a pair of cross-replicating enzymes depends on several factors, including their intrinsic catalytic activity, exponential growth rate with cognate substrates, ability to withstand inhibition by other substrates in the mixture, and net rate of production through mutation among the various cross-replicators. The A5B3 recombinant and its cross-replication partner B5′A3′ have different catalytic cores (Fig. 1C), and both exhibit comparable activity, accounting for their well-balanced rate of production throughout the course of exponential amplification (Fig. 3D). The selective advantage of this cross-replicator appears to derive from its relative resistance to inhibition by other substrates in the mixture (Fig. 3C) and its ability to capitalize on facile mutation among substrates B2, B3, and B4 and among substrates A2′, A3′, and A4′ that comprise the most abundant recombinants (fig. S2D).

Populations of cross-replicating RNA enzymes can serve as a simplified experimental model of a genetic system with, at present, two genetic loci and 12 alleles per locus. It is likely, however, that the

number of alleles could be increased by exploiting more than four nucleotide positions at the 5′ and 3′ ends of the enzyme and by relaxing the rule that these nucleotides form one G•C and three A•U pairs. In order to support much greater complexity, it will be necessary to constrain the set of substrates, for example, by using the population of newly formed enzymes to generate a daughter population of substrates (*9*). An important challenge for an artificial RNA-based genetic system is to support a broad range of encoded functions, well beyond replication itself. Ultimately, the system should provide open-ended opportunities for discovering novel function, something that probably has not occurred on Earth since the time of the RNA world but presents an increasingly tangible research opportunity.

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Antagonistic Actions of Msx1 and Osr2 Pattern Mammalian Teeth into a Single Row

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Mammals have single-rowed dentitions, whereas many nonmammalian vertebrates have teeth in multiple rows. Neither the molecular mechanism regulating iterative tooth initiation nor that restricting mammalian tooth development in one row is known. We found that mice lacking the transcription factor odd-skipped related-2 (Osr2) develop supernumerary teeth lingual to their molars because of expansion of the odontogenic field. Osr2 was expressed in a lingual-to-buccal gradient and restricted expression of bone morphogenetic protein 4 (Bmp4), an essential odontogenic signal, in the developing tooth mesenchyme. Expansion of odontogenic field in *Osr2*-deficient mice required Msx1, a feedback activator of *Bmp4* expression. These findings suggest that the Bmp4-Msx1 pathway propagates mesenchymal activation for sequential tooth induction and that spatial modulation of this pathway provides a mechanism for patterning vertebrate dentition.

Teeth are vertebrate-specific organs, and distinct dentition patterns have played critical roles in vertebrate diversification and specialization (*1*–*3*). In addition to variations in tooth number, size, and shape, many nonmammalian vertebrates have multirowed dentitions, whereas mammals develop teeth in a single row. Studies of tooth development in several fish species showed that multirowed dentitions result from sequential iterative tooth initiation along the mesial-to-distal and labial-to-lingual directions (*3*–*5*). The molecular mechanisms regulating the precise spatiotemporal patterns of sequential tooth initiation are unknown. Development of the single-rowed mammalian dentition likely involves restricting odontogenic field along the buccolingual axis; the mechanism underlying this control is also unknown.

Current understanding of the molecular mechanisms controlling tooth development has come mostly from studies in mice (*1*, *6*). Although supernumerary teeth have been reported in several mutant mouse strains (*7*–*10*), the majority developed within the tooth row from vestigial diastemal tooth buds (*10*, *11*). We recently generated mice lacking the *Osr2* (*odd-skipped related-2*) gene (*12*, *13*) and found that they exhibited supernumerary tooth development lingual to their molar teeth (Fig. 1, A and B, and fig. S1). Histological analyses (*14*) traced initiation of these supernumerary tooth germs to aberrant thickening of oral epithelium lingual to the first molar tooth buds at embryonic day 13.5 (E13.5) (Fig. 1, C and D). By E15.5, as the first molar germs developed to late "cap" stage (*1*), the ectopically thickened oral epithelia in *Osr2*$^{-/-}$ embryos invaginated and the underlying mesenchyme condensed (Fig. 1E). These ectopic epithelial invaginations resembled cap stage tooth germs by E16.5 (Fig. 1F). Because *Osr2*$^{-/-}$ mice die shortly after birth resulting from cleft palate (*13*), we transplanted E13.5 mandibular molar tooth germs under renal capsules of adult mice to allow complete tooth morphogenesis (*14*). After 21 days, wild-type and heterozygous molar tooth germs gave rise to two to three mineralized molar teeth, representing the normal molars (Fig. 1G). In contrast, *Osr2*$^{-/-}$ mutant molar tooth germs gave rise to four to five mineralized teeth (Fig. 1H and fig. S2). These data indicate that a complete odontogenic program was ectopically activated lingual to the molar teeth in *Osr2*$^{-/-}$ mice.

To gain insight into supernumerary tooth formation in *Osr2*$^{-/-}$ mice, we examined expression of selected marker genes during early tooth development. *Pitx2* was initially expressed throughout oral epithelium, and its expression selectively maintained in dental epithelium after E11 (*15*). In *Osr2*$^{-/-}$ embryos, *Pitx2* expression abnormally persisted in oral epithelium lingual to the first molar tooth buds (fig. S3, A and B). By E14.5, strong *Pitx2* expression marked the supernumerary dental placodes and first molar tooth buds (fig. S3, C and D). At E13.5, *sonic hedgehog* (*Shh*) was expressed in the enamel knot of developing molar tooth buds (*16*) (fig. S3E). In *Osr2*$^{-/-}$ mutants, *Shh* was ectopically expressed in a subset of epithelial cells lingual to the first molar buds (fig. S3F). By E14.5, *Shh* expression was clearly detected in the supernumerary dental placodes in *Osr2*$^{-/-}$ embryos (fig. S3H). In addition, expression of dental mesenchyme markers *Msx1* and *Lef1* were up-regulated and expanded lingually in *Osr2*$^{-/-}$ mice (fig. S3, I to L). These data suggest that supernumerary tooth development resulted from lingual expansion of the odontogenic field from the first molar tooth germs.

To understand how Osr2 regulates the odontogenic field, we examined *Osr2* expression during normal tooth development. At E11.5, *Osr2* was strongly expressed in the mesenchyme lingual to the dental lamina in both the maxilla and the mandible (Fig. 2A). *Osr2* was also highly expressed in the proximal mandibular mesenchyme buccal to the dental lamina (Fig. 2A). As tooth buds developed from E12.5 to E14.5, *Osr2* mRNA was expressed in a gradient in the developing tooth mesenchyme, with higher expression lingual and lower expression immediately buccal to the tooth buds (Fig. 2, B to D). Overall, the *Osr2* expression pattern is complementary to that of bone morphogenetic protein 4 (Bmp4) (Fig. 2, E to H), an essential odontogenic signal preferentially expressed on the buccal side in developing molar mesenchyme (*17*–*20*).

The expression pattern and mutant phenotype suggest that Osr2 functions to restrict odontogenic potential in the developing tooth mesenchyme. Consistent with this hypothesis, *Bmp4* expression was up-regulated and expanded into mesenchyme lingual to first molar buds in *Osr2*$^{-/-}$

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