1 **Modeling *Edar* expression reveals the hidden dynamics of tooth**

2 **signaling center patterning**

3 **Short title: the hidden dynamics of tooth patterning**

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30 **Abstract**

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32 The generation of patterns during development is generally viewed as a direct process. In the

33 mouse jaw, however, the sequential patterning of molars initiates with abortive tooth signaling

34 centers called MS and R2, thought to be vestiges of the lost rodent premolars. Moreover, the

35 mature signaling center of the first molar (M1) is formed from the fusion of two signaling centers

36 (R2 and early M1). Here, we report that *Edar* expression reveals the hidden dynamics of signalling

37 centers patterning. First, *Edar* expression evidenced a hidden two-step patterning process that we

38 modelled with a single activator-inhibitor pair: the epithelium is initially broadly activated, then

39 activation becomes restricted in space to give rise to the signalling centers. Second, *Edar*

40 expression unveils successive phases of pattern making and pattern erasing events, a phenomenon

41 that we called a developmental palimpsest. MS is erased by a broad activation for the benefit of

42 R2, which itself is erased before it recovers when the first molar signaling center forms. In the

43 lower but not the upper jaw, the two neighboring signaling centers then fuse into a single elongated

44 center. Our model recapitulated the erasure of the R2 signaling center by the wave of activation

45 that precedes the formation of M1 signaling center, and predicted the surprising rescue of R2 in

46 the context of an *Edar* mutant with reduced activation. It suggested that R2 was not intrinsically

47 defective, but actively outcompeted by M1 formation. We confirmed this by cultivating R2

48 separately from the posterior tissue and showing it could then generate a tooth. Finally, by

49 introducing chemotaxis as a secondary process of tooth germ maturation, we recapitulated the

50 fusion of R2 and M1 in the lower jaw only, and the loss of fusion when Edar function is impaired

51 in organ cultures. In conclusion, we have uncovered a highly indirect and dynamic nature of pattern

52 formation in the molar field that could nevertheless be simulated with simple mathematical

53   models. Our study argues for viewing embryonic patterns as dynamical objects rather than as fixed

54   endpoints, where dynamics is essential to the outcome of the patterning process.

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60 **Introduction**

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62 The emergence of ordered patterns in multicellular organisms has been a major field of research in

63 developmental biology, revealing a diversity of pattern formation mechanisms. While some patterns appear

64 simultaneously (e.g. drosophila segments, mouse hair), other appear sequentially (e.g. feathers on chicken's

65 back), most often as the structure grows distally (e.g. short-germ insects segments, somites, limbs proximo-

66 distal elements, palatal rugae). Several types of patterning mechanisms have been proposed, some relying

67 on a prepattern (e.g. "positional information" model: a gradient of a signaling molecule is turned into a

68 more complex pattern by interpreting the varying concentration at each position in space, (1,2)) and other

69 on spontaneous pattern formation (e.g. reaction-diffusion (Turing) mechanisms, chemotaxis, see below and

70 (3–5)). In these studies, more or less importance has been given to the temporal dynamics of pattern

71 formation depending on the mechanism. Sequential formation requires the consideration of temporal

72 aspects that can be neglected when the pattern forms at a glance (6,7). Spontaneous pattern formation

73 inherently emphasizes the dynamics of the system. Instead, positional information has been mostly

74 associated with static representations (e.g. the French flag model, see (3)). In all cases however, patterning

75 is viewed as a conceptually simple temporal process: from a prepattern or a spatial heterogeneity emerges

76 the final pattern. It is however questionable whether biological systems, which result from an historical,

77 contingent process, proceed in such a direct manner, or if transient patterns can be constructed and

78 deconstructed during embryogenesis until the final pattern is formed. Recently, the textbook example of

79 simultaneous pattern formation, i.e. the formation of *Drosophila* gap gene expression pattern, was closely

80 reexamined, and it was found that the gene expression pattern was changing with time, as maternal inputs

81 decay, with important consequences for the final pattern (8). To our knowledge, other examples are lacking.

82 Here, we studied this question in a model of sequential patterning: the mouse molar row.

83 The search for the general mechanisms generating patterns in biology has been greatly influenced by the

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84   theoretical work of the mathematician Alan Turing (4,5,9). The generalization of this work has led to many

85   classes of reaction-diffusion (RD) mechanisms, where two (or more) molecules characterized by different

86   spatial range of action and a given topology of interaction can self-organize a stable pattern, but also exhibit

87   behaviors such as oscillations or propagating waves (4). The most iconic example is the case where a short-

88   range activator that self-amplifies and activates its own long-range inhibitor, can create spots, stripes or

89   labyrinths. Recently it has been shown that many biological systems exhibit features of RD mechanisms

90   (e.g. patterning of epithelial appendages such as hair (10,11), the patterning of features such as the rugae

91   of the palate (12), digits (13) and somites (14)). This should not be taken too strictly however. Geirer and

92   Meinhardt pointed out that any process involving local self-enhancement and lateral inhibition has the

93   potential to drive spontaneous pattern formation (15). For example, color pattern formation in zebrafish

94   can be explained by RD models, but at least partly involves cell interactions rather than the diffusion of

95   biomolecules (16,17). Pattern formation can also arise from purely chemostaxis mediated self-organization.

96   When cell movement is driven by concentration gradients of chemostatic cues, positive feedbacks between

97   cell density and chemo-attractant production are known to enhance local concentration of cells and may

98   result in self-sustained aggregation. (18–20). Chemotaxis plays a prominent role in feather formation (21),

99   and this is likely also the case in most other epithelial appendages (e.g. hair (Glover et al., 2017)).

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101   Mouse molars are a good example of repeated structures that form through sequential pattern formation.

102   Mice have only three molars per quadrant, separated from incisors by a diastema, the other mammalian

103   teeth (i.e. canine and premolars) having been lost in the evolution of mouse lineage (22). Molars develop

104   sequentially from the most anterior molar (first molar, M1) to the most posterior (third molar, M3). They

105   develop from a unique cylinder-shaped invagination of the oral epithelium, the so-called dental lamina,

106   (23–25) where tooth-specific signaling centers, called Primary Enamel Knots (PEK) are patterned. These

107   signaling centers then drive the formation of individual teeth by promoting "cap" formation; the capping

108 of the underlying condensed mesenchyme by the epithelium. Indirect evidence that activation-inhibition

109 mechanisms determine sequential formation of these signaling centers comes from the similarity of tooth

110 formation with other epithelial appendages (26), namely hair and palatal rugae, whose patterning in clearly

111 ruled by Turing-type mechanisms ((11,12)). The most direct evidence is a study by Kavanagh and

112 colleagues (27), showing that when tissue that will form M2 is separated from M1, M2 forms earlier and

113 becomes larger. PEK formation in the epithelium requires signaling from both the epithelium and the

114 mesenchyme (28), including a mechanical signal induced by mesenchyme condensation (29).

115 The sequential patterning of mouse molars in the lower jaw (Figure 1A) involves two transient

116 signaling centers (30), that fail to drive proper cap transition, yet form morphologically

117 distinguishable buds (30,31). These buds might be the vestige of lost premolars (30,32).

118 Monitoring these signaling centers *via Shh* expression has shown that the signaling center called

119 MS initiates sequential patterning and then disappears (30). Subsequently, the R2 signaling center

120 forms. As it vanishes, the M1 early signaling forms posteriorly (Prochazka et al., 2010). Soon

121 after, the former R2 and M1-early signaling centers are encompassed in a giant *Shh*-expressing

122 signaling center (Prochazka et al., 2010; Lochovska et al., 2015). Here, we will refer to it as the

123 mature M1 signaling center. A similar situation with two abortive buds (called R1 and R2) has

124 been noticed in the upper jaw (25). Their signaling centers have not yet been characterized,

125 although they are morphologically more apparent than in the lower jaw.

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Figure 1: *Edar* expression is dynamically regulated during hair and tooth patterning

A- Scheme summarizing the sequential patterning of signaling centers in the dental lamina as revealed with *Shh* expression.
B- Scheme showing *Edar* regulation in epidermis during primary hair patterning. Concomitantly with hair placodes patterning, *Edar* becomes upregulated in the hair placode while being downregulated in its neighborhood, highlighting the outcome of the activation-inhibition mechanisms that patterned the placode

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134   and avoid further formation of placodes in the vicinity.

135   C- *In situ* hybridization showing *Edar* expression in the isolated dental epithelium from 14.5 to 16.0 days, a
136   period corresponding to growth of the tail region ("M2 forming region") and patterning of M2 signaling
137   center in this newly grown region (at 16.0). At 14.5, *Edar* is restricted to the primary enamel knot of the first
138   molar (yellow on the scheme). At 15.0, *Edar* expression is seen in the posterior-most part of the tail, the M2-
139   forming region (Gray on the scheme). Between 15.5 and 16.0, it is restricted to the primary enamel knot of
140   the second molar (M2, green on the scheme). On the scheme, the continuing expression of *Edar* in the
141   maturing first molar has been omitted for simplicity.

142   D- Scheme summarizing the expression pattern in C, to be compared with B. The situation in hair is similar to
143   the situation in the anterior part of the dental epithelium.

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146 Interestingly, mutations in genes affecting various developmental pathways (FGF, Shh, Wnt, BMP and

147 Eda pathways) lead to a supernumerary tooth in front of M1, thus resembling a premolar (34). Where it

148 was specifically studied, the results were consistent with R2 signaling center being enabled to form a tooth

149 (35–39,33). The picture is thus fairly complex, especially since we lack direct evidence for the dynamics

150 of activation-inhibition mechanisms that pattern signaling centers in the dental lamina and promote tooth

151 formation.

152

153 The Eda pathway has the potential to shed light on these mechanisms. This pathway shows a consistent

154 role in activation-inhibition mechanisms throughout several epithelial appendages (hair, feather, teeth;

155 (40,41). This role has been more specifically evidenced for mouse guard hair pattern formation. The

156 receptor of the pathway, Edar, is first broadly expressed in the epidermis. Concomitantly with hair signaling

157 center patterning, it becomes upregulated in the placodal signaling center, and downregulated in the

158 neighborhood (Figure 1B). In the absence of Edar signaling, no signaling center forms (11,42,43) , while

159 increasing signaling results in more numerous and densely packed placodes (11). Current models posit that

160 the Eda pathway is activated by Wnt, activin BA and BMP4 pathways (43,44) (11,44), but also feeds back

161 on these pathways (and others) through the transcriptional activation of their diffusing ligands and

162 inhibitors (*e.g.* WNT10a/b, DKK4, CCN2/CTGF, Follistatin and FGF20 (40,41)). More recently, it

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163   appeared that Eda signaling also promotes placodal fate by stimulating the centripetal migration of cells in

164   the epithelium (45,46). In teeth, the Eda pathway is dispensable for primary signaling center (PEK)

165   formation, but required for its correct sizing (46–48). Similarly, it is necessary for correct patterning of the

166   secondary signaling centers controlling cusp morphogenesis (38,47,49). *Eda* and *Edar* mutants have

167   reduced tooth size and cusp number, but intriguingly, sometimes have a small supernumerary tooth (50–

168   52). In gain-of function mutations, an anterior supernumerary tooth is also found, and teeth are larger with

169   more cusps (49,53–55).

170

171   In this paper, we aimed at clarifying the temporal dynamics of signaling center formation in the dental

172   lamina. We studied the temporal dynamics of *Edar* gene expression, the receptor of the Eda pathway,

173   during molar pattern formation and showed it recalls the dynamics observed during hair patterning. Based

174   on these data, we built a reaction-diffusion type model of molar patterning that enables sequential signaling

175   center formation and helps reveal the exquisitely complex temporal interactions leading to the construction

176   and deconstruction of patterns in the developing molar row. Our model explains a counter-intuitive result,

177   the rescue of the abortive R2 bud in the context of reduced activation/increased inhibition of the Edar loss

178   of function mutation. Finally, we show that Edar is necessary for the formation of a fused R2-M1 signaling

179   center in the lower jaw only, possibly through a chemotactic effect. We thus showed that patterning is not

180   direct, although it follows simple mathematical rules.

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181 **Results**
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183 ***Edar* regulation highlights activation in the growing dental epithelium**

184 To get insights into molar row patterning, we examined the regulation of the *Edar* gene (Figure 1B).

185 Because the early period of molar row patterning is complicated by the presence of vestigial signaling

186 centers, we first focused on the patterning of the second molar. Since *Edar* is exclusively expressed in the

187 epithelium, we performed *in situ* hybridization on mandibular epithelium that has been dissociated from

188 the mesenchyme, thus providing a 3D view of *Edar* expression. At 14.5 dpc, *Edar* expression is restricted

189 to the primary signaling center (PEK) of the first molar, and no expression is seen in the second molar field,

190 looking like a "tail" (Figure 1C). At 15.0 dpc, the "tail" has elongated and *Edar* expression is upregulated

191 in the posterior most part of it. By late 15.5 dpc, it starts restricting to the M2 primary enamel knot, just

192 before M2 cap transition occurs (at 16.0 dpc). The restriction was concomitant with *Shh* expression starting

193 in M2 PEK (data not shown and (30)).

194 This dynamic of an initial broad upregulation of *Edar* followed by its restriction to a signaling center is

195 reminiscent of what happens during hair patterning (compare Figure 1B and 1D). It suggests that the

196 decision to form a tooth signaling center in the growing molar field proceeds in two phases. First, the whole

197 dental epithelium is activated. This activation results in broad *Edar* expression, so far the only gene to show

198 such an expression pattern marking the epithelium competent to form a tooth. Second, activation gets

199 restricted spatially and gives rise to a signaling center. This results in the focused expression of Edar, and

200 many other genes known as primary enamel knot genes (*e.g. Shh*). In this view, *Edar* expression is a read-

201 out of activation levels in the molar field: where it is high enough, *Edar* is expressed. To further formalize

202 these ideas, we built a mathematical model of activation in the dental epithelium, as followed by *Edar*

203 expression pattern.

204

205 **Activation dynamics can be modeled by a transition from a bistable system to a Turing system in a**

206 **growing domain**

207 Activation (monitored by Edar expression) can switch between different states: from no activation (that is

208 no *Edar* expression) to broad activation (broad *Edar* expression) and from broad activation to spatially-

209 restricted activation (focused *Edar* expression) suggestive of Turing mechanisms in the dental lamina.

210 From a mathematical point of view, these complex behaviors can be modeled as two regimes of the same

211 reaction-diffusion system (Figure 2A). The first regime, named throughout this paper *the bistable regime*,

212 describes solutions connecting two constant, stable states (respectively, a high activation state and a low

213 (no) activation state). This corresponds to the anterior part of the tissue with transient up-regulation of

214 *Edar*. Second, the so-called Turing regime, differs from the previous one by the modification of one

215 parameter (auto-inhibition strength). It is characterized by stable heterogeneous patterns which emerge

216 from homogeneous patterns (*e.g.* spots). Thus the same system of equations describing the interaction of

217 an activator and its inhibitor models waves of activation in the dental lamina as well as its restriction to the

218 signaling center. A simple change in one reaction parameter could switch the system from the bistable

219 region to the Turing regime. Because the restricted expression is only seen in the developmentally advanced

220 parts (i.e. the anterior part) and follows broad activation, we impose that the activator has a positive

221 feedback on tissue maturation, resulting in the switch to Turing regime. Biologically, this means that upon

222 the broad wave of activation, new gene products have been produced that modify the activation-inhibition

223 parameters.

224

225 **Figure 2: a model for signaling center patterning based on maturation-dependent shift from**
226 **a bistable to a Turing regime**
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228 A- Basic principles for a mathematical model of activation in the dental field, with *Edar* expression considered
229 as a read-out of activator concentration. The dental epithelium first transits from no activation to generalized
230 activation, as the tissue is primed by the anterior mesenchyme. Activation induce tissue maturation, moving
231 the system to a Turing regime, which needs a single parameter change. Activation is localized to the signaling

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232   center.
233   B- We modeled activation-inhibition mechanisms along the antero-posterior axis (1 dimension). Activator
234   concentration is shown through space (x) and time (y), as the domain grows. Snapshots taken at three
235   different timepoints are shown (red: activator concentration, blue: inhibitor concentration, green: domain
236   maturation). The simulation shows the periodic behavior of the model: The dental epithelium grows in an
237   inactivated state, due to inhibition coming from the Turing spot (snapshot 1, see also Figure 1 C, 14.5 dpc).
238   Newly grown parts of the dental epithelium get activated on a periodic basis (bright yellow anterior domains,
239   snapshot 2, figure 1C 15.0-15.5 dpc), and upon maturation, produce a Turing peak (snapshot 3, Figure 1C
240   16.0 dpc). The movie corresponding to the snapshots is available as supplementary material 2.
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242

243   Based on the above assumptions, we built a one-dimension model of RD mechanisms in an oriented

244   growing domain. The model describes the time evolution of concentration of an activator and its inhibitor,

245   which diffuse with different speeds and undergo kinetic reactions with explicit parameters regulating the

246   transition between Turing and non-Turing regimes (Figure 2A). A detailed description and the parameters

247   used in all simulations are found in the supplementary material (Supplementary Material 1). Below we

248   summarize the main characteristics of the model, that exhibits periodic behavior, as shown in a

249   representative simulation (Figure 2, see also the movie as Supplementary material 2).

250   The tissue grows from its anterior end, and the newly produced tissue matures exponentially in time (albeit

251   at a slow rate). Maturation is stimulated in presence of the activator, with a certain time delay. In zones

252   where maturation reaches a threshold value, the system parameters irreversibly switch from a bistable to a

253   Turing regime (Figure 2B, snapshots 2 to 3).

254   Before this switch can occur, such simple system first needs to reach high levels of broad activation in the

255   newly grown part (Figure 2B, snapshot 2). Based on the literature, epithelia-mesenchyme interactions may

256   play an important part in this broad activation (see Discussion), but the mechanism is largely unknown.

257   Here, we simply assumed an extrinsic component representing the interaction with the mesenchyme. Below

258   a certain threshold of activation, it will act to increase the concentration of the activator. Above a certain

259   threshold, it will feedback negatively on it. This introduces an oscillatory behavior at the anterior end of

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260   the domain. Interestingly, although we do not explicitly put this in the model, the oscillatory behavior of

261   the mesenchyme is spatially coupled to the domain growth: the transition from "no activation" to

262   "activation" is promoted by the positive feedback from the mesenchyme, but will only happen when the

263   domain has grown enough to escape from the influence of the inhibitor from the Turing peak (Figure 2B,

264   snapshot 1 to snapshot 2).

265   In summary, our theoretical model based on *Edar* expression involves activation-inhibition mechanisms in

266   the dental epithelium, coupled with periodic activation of the growing dental epithelium.

267

268   **A developmental palimpsest occurs for MS and R2 patterning and can be modeled with a regime of traveling wave**

269   Next, we focused on the dynamics of *Edar* expression during the complex chain of patterning events

270   (schematized in 1A), that precedes the formation of the M1 signaling center, also known as the PEK (yellow

271   in 1A/1C). The dynamics was partly similar to that observed for M2 patterning, although MS and R2

272   signaling centers fail to drive cap formation and to form a tooth (Prochazka et al. 2010 and figure 2A).

273   Indeed, broad *Edar* expression restricts to these signaling centers, in late 12.5 embryos and early 13.5

274   embryos, respectively (corresponding to Shh-signaling center at 12.7 and 13.3 dpc in Prochazka et al. 2010

275   and figure 2A). Following the restriction, *Edar* expression starts again from the anterior part of the dental

276   epithelium (white arrowhead). However, contrary to the situation in M2, for which the wave of *Edar*

277   expression stops at a distance from the M1 area (figure 1C, 15.0 dpc), in both cases, it invaded the whole

278   dental epithelium including its anterior most part, thus erasing the previous Turing pattern, made by MS

279   and R2 signaling centers (figure 3A and 3B 12.5 dpc and figure 3C 13.5 pdc). In the case of MS, a new

280   signaling center is formed following the wave: it is R2 signaling center (according to DiI tracing in

281   Prochazka et al. 2010, it is just slightly posterior to MS). In the case of R2, two signaling centers are seen

282   following the wave: R2 recovers and the early M1 signaling center is newly formed (although soon later,

283   a single domain is formed see Fig 3C, 14.0 dpc, this will be discussed in detail below). We call this

13

284 phenomenon a developmental palimpsest, because a palimpsest is a manuscript page that has been scraped

285 or washed off to be used again for a novel text: here, a first Turing pattern (a first text) is erased by the

286 *Edar* expression wave (text scraping) and a new pattern is formed (novel text).

287
288 **Figure 3: Pattern erasing ("developmental palimpsest") in the early dental epithelium can be modeled with a**
289 **travelling wave**
290 A, B, C - *in situ* hybridization with an *Edar* probe of whole mandibles (only one half is shown, A) or isolated
291 dental epithelia (B, C) from 11.5 dpc to 14.5 dpc. Pictures are shown with an accompanying scheme where
292 *Edar* broad expression is shown in grey and *Edar* focused expression is shown in color. Several rounds of
293 *Edar* regulation are observed, culminating with the formation of a signaling center. The first pattern (MS
294 signaling center, A) is erased by a new wave of *Edar* expression (late 12.5-13.0), resuming in a second
295 pattern (R2 signaling center, B, D), that in turn is erased by another wave of *Edar* expression (13.5). A
296 pattern with 2 spots is transiently observed that finally resumes in a large spot corresponding to M1 signaling
297 center (C, D).
298 D Pattern erasiure can be observed in a modified model, where we introduce an asymmetry in the bistable
299 regime. The left panel shows the chronogramme of a representative simulation (concentration of activator
300 as a function of space (x) and time (y). Snapshots taken at three different timepoints are shown (red: activator
301 concentration, blue: inhibitor concentration, green: domain maturation). Snapshot 1 corresponds to R2 peak
302 (13.0 dpc in panel B). Snapshot 2 shows the activation wave invading R2 domain (13.5 dpc in panel C).
303 Snapshot 3 shows recovery of the R2 peak, together with the newly formed M1 peak (early 14.0 dpc in
304 panel C). The movie corresponding to the snapshots is available as supplementary material 3.
305

306

307 In our model, we could reproduce this behavior by imposing to the immature dental epithelium a bistable

308 regime with inactive and active stable states. This is a generic reaction-diffusion mechanism for wave

309 propagation. A representative simulation of this modified model is shown in Figure 3D (see also the movie

310 as supplementary material 3). The active state is more stable, so that once activation is primed in the anterior

311 part, a wave activates progressively the inactive area (Figure 3D, snapshot 2). It is important to notice that,

312 before wave initiation, the immature area is maintained naturally in the (less) stable inactive state under

313 the influence of the Turing initial peak. The wave can even propagate into the mature tissue and erase the

314 previously formed Turing pattern (Figure 3D, snapshot 1 and 2). Then, as a consequence of tissue

315 maturation subsequent to wave activation, two activation peaks are formed by a secondary Turing

316 patterning (Figure 3D, snapshot 3). This would correspond to R2 recovery and the newly formed M1

14

317   signaling center. For this palimpsest to occur, the wave that initiates in the immature (bistable) domain

318   should interact with the stable pattern in the mature Turing domain. Both the activation wave and the Turing

319   peak feature stability. As such, understanding their interaction is far from trivial. Several conditions must

320   be fulfilled in order to observe a palimpsest in the numerical tests, which are reviewed in Supplementary

321   Mat S3. In particular, we found that auto-inhibition, if increased in the bistable regime, strengthened the

322   wave and favored the palimpsest. We also found that it is sensitive to the temporal dynamics. It requires a

323   suitable synchronization between domain growth, anterior activation, wave speed, and maturation rate.

324

325   **Increasing inhibition in the model can explain the counter-intuitive rescue of R2 signalling center in**

326   *$Edar^{DlJ}$* **mutant**

327   Most of the numerous mutants where a premolar-like tooth forms, supposedly from R2, have larger or

328   simply normally sized molar teeth. The teeth of loss of function mutants for the Eda pathway are poorly

329   grown, yet a premolar-like tooth can form. A rescue of R2 is counter-intuitive in such context, therefore,

330   we decided to re-examine one of these mutants (*$Edar^{DownlessJ}$*, abreviated *$Edar^{DlJ}$*) in the light of *Edar*

331   dynamics and the present model.

332   First, we looked at the dynamics of *Edar* expression in *$Edar^{DlJ}$* mutant, to check if R2 was indeed rescued

333   in this mutant. This mutant encodes for a defective Edar protein due to a single amino-acid change, but the

334   gene is still transcribed. In contrast with the mutant epidermis, in which *Edar* regulation is lost (*Edar*

335   expression stays at uniform low levels in the epidermis) and hair fails to form (56), we still observe *Edar*

336   restriction to tooth signaling centers (Figure 4A), consistent with teeth being formed. However, the

337   dynamics of activation-inhibition mechanisms was modified in this mutant. We observed high variability

338   between embryonic tooth rows (including left/right), in line with the high phenotypic variability seen in

339   adults with losses of function for the Eda pathway (teeth rows with 2 or 3 or in rare cases 4 teeth) (50–52).

340   In the lower jaw (Figure 4A), we did not find obvious differences early in 12.5 dpc *$Edar^{DlJ-/-}$* embryos as

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341   compared with $Edar^{DlJ+/+}$ embryos, all of them exhibiting restriction of *Edar* to MS signaling center that is

342   also stained by *Shh* expression (not shown). In both cases, wild type and mutant, *Edar* expression is next

343   found in the whole dental lamina (13.0 dpc, Figure 4A). However, no restriction was observed in $Edar^{DlJ-}$

344     $^{/-}$ 13.5 dpc embryos as normally seen in their wild type counterpart (FVB background). Homogenous *Edar*

345   expression was still observed in most 14.0 dpc $Edar^{DlJ-/-}$ embryos at a time when homogenous *Edar*

346   expression is again observed in $Edar^{DlJ+/+}$ embryos. From 14.5 dpc, a restriction to a signaling center was

347   observed in most embryos (here named T1 PEK, with or without expression in the "tail"), while others still

348   display more or less continuous *Edar* expression. We noticed that this signaling center in the mutant is

349   found more posteriorly in the jaw than is the R2 signaling center (Figure 4B). At 15.0 dpc, we see either

350   one signaling center with *Edar* expression in the tail or two signaling centers (named T1 and T2). Possibly

351   the later case is due to approximately simultaneous patterning of two signaling centers from a dental

352   epithelium that was showing continuous expression in the previous stage. At 15.5, T1 has developed into

353   tooth germ of different sizes, from a very small one to a tooth germ equivalent in size to T2. To conclude,

354   our results show that R2 patterning is both postponed and displaced posteriorly in the $Edar^{DlJ-/-}$ mutant and

355   the resulting signaling center persists to form a tooth germ of variable size. Why then is R2 rescued in the

356   context of poorly grown teeth?

357

358 Figure 4: the changes in signaling center patterning observed in the $Edar^{DlJ}$ mutant are predicted by
359 the model

360   A- In situ hybridization with an *Edar* probe in dissociated lower jaw epitheliae from $Edar^{DlJ/DlJ}$ mutant and its
361   wildtype background (top views), for embryos of similar weight class between 13.0 and 15.5 dpc. Patterning
362   of the first signaling center (MS) is correct (not shown), but patterning of the second signaling center (R2,
363   here called T1 in the mutant) is delayed with variability (embryos >200mg). This T1 signaling center is not
364   erased and persists. Inc: incisors.
365   B- A side view of pictures outlined in red in A, showing that T1 signaling center is found more anteriorly in the
366   invaginated dental epithelium of the mutant as compared to the wild type.
367   C- Same simulation as in figure 3, but with increased inhibition. The position and timing of Turing peak
368   formation in wild type (as in figure 3E) are indicated with red dashlines. Increasing the inhibition (lowering
369   auto-inhibition) is sufficient to abolish the palimpsest and stabilize the first and the second peak.

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372   We next used our model to explain this non-intuitive observation. Eda pathway loss of function has been

373   shown to increase inhibition in other appendages (Mou et al. 2006; Harjunmaa et al. 2014), which is

374   consistent here with T1 signaling center being patterned further away from the anterior end of the dental

375   epithelium. Therefore, we analyzed numerically the effect of stronger inhibition (by decreasing auto-

376   inhibition rate). Interestingly, this was sufficient to recover qualitative behaviors consistent with the data:

377   1- T1 and T2 are formed later than R2 and M1 (red dashed line). 2- T1 is displaced anteriorly (blue dashed

378   line) 3 – the wave no longer destabilizes T1: a Turing pattern is formed that is not subjected to a palimpsest.

379   These results suggest two things. First, that a new wave of activation associated with the formation of the

380   next signaling center will naturally destabilize any pre-existing signaling center, if inhibition from this pre-

381   existing center is weak enough. Second, because we do not impose any difference between signaling

382   centers, this shows that R2 is not intrinsically defective, but actively outcompeted by the activation wave

383   associated with M1 formation. This is a major output of our modeling effort but it is in contradiction with

384   the previous hypothesis, in which the R2 signaling center is considered to be intrinsically defective.

385   Therefore, we decided to directly test this hypothesis.

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389 **The anterior part of the dental lamina (R2) is capable of forming a tooth - if separated from the**

390 **anterior part.**

391 If the anterior part of the dental epithelium is intrinsically defective for tooth formation, it should not be

392 able to give rise to a fully develop tooth when removed from the early M1 signaling center. On the contrary,

393 if the anterior part is not intrinsically defective, but normally outcompeted by the M1 as we suggest, a tooth

394 should be able to develop from it when removed from M1 influence. To test this, we cut the anterior part

395 (R2 part) from the anterior part (early M1 signaling center and anterior tail). As expected from our

396 predictions, the anterior part developed into a fully growing tooth. Remarkably, the timing of development

397 is advanced compared to the anterior part by 1 day, in accordance with the R2 signaling center having been

398 patterned earlier than the M1 one (Figure 5).

399 Taken together, these results are consistent with a model in which the R2 region is fully competent for

400 tooth formation, but is actively outcompeted by the forming M1, resulting in the developmental palimpsest

401 effect described.

402

403 **Figure 5: The anterior part of the molar field corresponding to R2 signaling center can develop to a**
404 **tooth germ when separated from the anterior part**
405 Left: The developing molar region was dissected from 14.3 dpc embryos and the anterior most part corresponding
406 to R2 signaling center was separated from the rest, including the early M1 signaling center and the anterior "tail".
407 The following day, a signaling center was recovered in the anterior part and formed a tooth germ (6 days cultivation).
408 After two days, a signaling center formed in the anterior part and formed a similarly sized tooth as the anterior part.
409 In the control experiment (right), the second molar does not form before the third day.

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18

The formation of a large signaling center depends on Edar activity

412

413 We then focused on another feature of the mouse dental row: the incorporation of R2 into M1 which is

414 hypothesized to play a crucial role for the formation of the anterior part of M1, both during development

415 and evolution (30,33). This corresponds to another curious behavior of *Edar* expression dynamics: the

416 fusion of R2 and early M1 signaling centers soon after recovering from the palimpsest (Figure 3C).

417 We examined the 13.5-14.5 dpc period, which corresponds to M1 PEK formation, in detail. To do so we

418 followed in parallel the dynamics of *Edar* expression, *Shh* expression (a recognized marker of tooth

419 signaling centers) and Wnt pathway activity (monitored by the TOPGAL reporter) (Figure 6A). In late 13.5

420 dpc/ early 14.0 dpc embryos, *Shh* expression and TOPGAL X-gal staining reveals that the M1 signaling

421 center starts to form. Some faint *Shh* expression is occasionally seen in R2, while X-gal staining persists

422 there, presumably in part due to B-galactosidase long half-life. At this stage, *Edar* is also focused in R2

423 and M1 signaling centers, yet low expression can also be seen around. In slightly older embryos, robust

424 *Edar* expression is seen in a domain spanning the two signaling centers, and aligned with the barely formed

425 cervical loops. This *Edar* expression is followed by anterior expansion of *Shh* expression, that finally spans

426 the position of former R2 and early M1 signaling centers (as shown in Prochazka et al. 2010). We also

427 observed upregulation of TOPGAL activity during the same period. All together, these results show that

428 R2 and early M1 signaling centers are re-patterned as a single large signaling center highlighted by Edar

429 expression. This early event prefigures TOPGAL activity and *Shh* expression relocalization in a large

430 signaling center.

431

432 **Figure 6: The formation of a large fused PEK depends on Edar signaling, possibly through**
433 **chemotaxis**
434 **A,F:-** Dynamics of *Edar* (upper) and *Shh* (lower) expression and TOPGAL reporter X-gal staining (middle)
435 during the 14.0 to 14.5 dpc period when M1 PEK forms, in lower (**A**) and upper (**F**) jaw of CD1 mice. In
436 both jaws, *Edar* expression spans both R2 and M1 region before becoming restricted. In upper jaw, and very
437 transiently in the lower jaw, expression restricts again to R2 plus the newly formed M1 signaling center
438 (220-240 mg and 180-190 mg, respectively). In lower jaw, expression restricts to a large domain including

19

439　both R2 and early M1 signaling center (from 220-230 mg). *In situ* hybridization with *Edar* or *Shh* probes
440　and X-gal stainings were performed on dissociated lower or upper jaw epitheliae. The embryonic weights
441　used to stage embryos are shown. Staining concentrated in R2 signaling centers is pointed with a white
442　arrowhead, while M1 signaling centers are pointed with a yellow arrowhead.
443　**B -**Lower molar rows were put into culture at 13.0 dpc and following 2 hours of recovery, they were treated
444　with an Eda blocking antibody (EctoD3**)** or with mock for 40h. The dissection process tends to interfere
445　with the formation of a large signaling center: in most samples, low *Shh* expression is seen between R2 and
446　early M1 signaling center; however, R2 expression is always maintained. In EctoD3-treated samples, R2
447　expression is lost and only a small posterior M1 signaling center is found.
448　**C, D, E, H** – Numerical simulation of a simple Turing model augmented with chemotaxis in a fixed, matured
449　domain with a simple configuration consisting in two Turing peaks **C:** in absence of chemotaxis (χ=0), two
450　Turing peaks form in a domain of length L=2.6. **D, E:** in a model where the activator stimulates the
451　production of a chemoattractant (over a certain threshold), Turing peaks form first, and later chemotaxis
452　fuse the two peaks in a single large peak (χ=0.5, E). However, the fusion requires sufficient activity of the
453　chemoattractant (χ=0.1, D). **H** On a larger domain (L=3), a Turing pattern with a larger wavelength is
454　chosen, similar levels of chemotaxis as in D (χ=0.5) are no longer sufficient to fuse the peaks.
455　**G** – Distances between R2 and M1 signaling center as measured on TOPGAL dissociated epithelia at 14.0
456　dpc in lower and upper jaw. R2 and M1 signaling centers form closer in lower jaw than in upper jaw.
457
458

459　Because *Edar* expression prefigures the anterior expansion of *Shh* expression domain and *Edar* has been

460　shown to regulate *Shh* (42,57,58), we wanted to test if Edar signaling is necessary for anterior expansion

461　and the formation of a large M1 PEK. To specifically test this, we dissected 13.0 dpc lower molar regions,

462　when R2 has already formed, and cultured them for 48h with or without an interfering antibody, so that we

463　knock-down Edar signaling in the next period of M1 PEK formation. We then visualized *Shh* expression

464　on isolated epithelia. In untreated samples, we occasionally observed a large M1 PEK similar to *in vivo*

465　samples (Figure 6B, state 0), but most often it was split in two spots (corresponding to R2 and an extended

466　early M1 expression) bridged by a narrow domain of *Shh* expression (Figure 6B, state 1). This can be

467　explained by the fact that the dissection process could change the activation-inhibition balance in favor of

468　inhibition (as proposed in (59)), which according to the predictions of our model, should favor R2

469　persistence. In treated samples, we mostly recover a small, very posterior signaling center, which

470　corresponds to early M1 *Shh* expression (Figure 6B, state 2 and 3). Moreover, the dental epithelium is

471　morphologically different, showing a bud, followed by a small cap and a "tail". Thus, in the absence of

472　Edar signaling, *Shh* expression is lost in the R2 region and only a small PEK forms, which is equivalent in

473   size and position to the early M1 signaling center, and which drives cap transition there. Taken together,

474   our results show that Edar signaling is essential for the formation of a large PEK that encompass R2 and

475   early M1 signaling centers.

476

477   **Coupling chemotaxis to the Turing system reproduces biological variability in signaling center fusion**

478   Recent studies have pointed out that chemotaxis may plays a role in the formation of tooth and hair placodes

479   (10,45,46) and the Eda pathway activated centripetal migration in the placodal epithelium (45). We noticed

480   that the TOPGAL stainings tend to contract in the antero-posterior direction as the M1 signaling center

481   matures and the distance between R2 and early M1 signaling center decreases (compare 14.0 and 14.5 dpc

482   samples in figure 6A). This suggested us that cell movements may take part in the formation of the large

483   signaling center.

484   To evaluate this possibility, we incorporated cell motion through chemotaxis in a simple Turing system

485   producing two peaks, thus starting with the situation when R2 and M1 signaling center co-exist (Figure 6

486   C). We assumed that the chemoattractant pattern corresponds to the activator pattern (*e.g.* cells move

487   towards regions of higher activator concentration). Cell aggregation mediated by chemotaxis requires a

488   positive feedback loop between cell density and chemoattractant concentration (18,19). In our setting,

489   without addition of extra molecular entities, this feedback loop can act either directly on the activator-

490   chemoattractant (as in the classical Keller-Segel system, (19)), or *via* down-regulation of the inhibitor (*e.g.*

491   higher cell density affects negatively inhibitor concentration). The latter configuration produced the

492   expected behavior: signaling centers form and secondarily they fuse in a single large signaling center

493   (Figure 6E). This is consistent with the intuitive idea that fusion requires sufficiently long range

494   communication between the two Turing peaks, and thus a feedback on the long-range diffusing species (in

495   our case the inhibitor). Reducing chemotaxis efficiency resulted in the lack of fusion, consistent with our

496   experiments reducing Edar signaling, and presumably, chemotaxis (Figure 6D).

21

497   Nonetheless, adding chemotaxis to our system does not necessarily result in the fusion into a single spot.

498   In our *in silico* simulations, we observed that the transition between fusion and the absence of fusion

499   depends on various parameters. The reason is that the activator-chemoattractant has a direct positive effect

500   on the inhibitor, but an indirect negative effect on the same inhibitor by means of cell recruitment. These

501   ambivalent effects make chemotaxis able to compensate the segregation due to Turing patterning in some

502   situations, or reinforce it in other situations. For example, we observed that chemotaxis can favor pattern

503   formation where the Turing system fails to produce a pattern alone. This suggests us that chemotaxis may

504   be part of the normal formation of tooth signaling centers, even when they stay separated.

505   In line with this, we noticed that there is no fusion in the upper jaw (Figure 6F), where the distance between

506   signaling centers is initially larger by about 30% (Figure 6F and G). A small increase of the domain size,

507   as seen between lower and upper jaw, increased the Turing wavelength and was sufficient to abolish the

508   fusion under the same chemotaxis efficiency (Figure 6H, a 15% increase is sufficient with our parameters

509   setting).

510   In conclusion, the non-trivial interaction between chemotaxis and a Turing system appears to be a plausible

511   mechanism to explain the variability in the dynamics of tooth signaling centers in lower jaw, upper jaw

512   and various mutants, including *Edar* loss of function.

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515 **Discussion**

516

517 In this study, we have revealed the highly complex and dynamic behavior of signaling centers responsible

518 for tooth patterning in the mouse jaw. Patterning is usually seen as a directional process, rather than a

519 dynamic process that could take circuitous routes. However, we show that patterning of the first molar

520 involves what we called a developmental palimpsest, where patterns are established, erased or remodeled

521 to give rise to new patterns. Using a mathematical approach, we show that these behaviors (pattern erasing,

522 recovery, rescue, fusion) despite seeming to be complex can be be produced by the activity of simple

523 mechanisms (a Turing pair with two regimes, as well as chemotaxis).

524

525 **From similarities in *Edar* expression dynamics to differences between hair and tooth patterning**

526 In this study, we have revealed the highly dynamic expression of *Edar* in the developing molar row. This

527 dynamic is superficially similar to that seen during hair patterning. This is not surprising since hair and

528 tooth patterning share many common features (Mikkola, 2007; Biggs and Mikkola, 2014), making their

529 comparison highly instructive. Below, we compare these two systems in light of our results.

530 In teeth, as in hair, *Edar* expression becomes restricted to the signaling center as it is patterned. We have

531 noticed however two substantial differences: i) In skin, the initial basal levels of *Edar* are upregulated in

532 the placode and downregulated in its vicinity. This is thought to be pivotal for placode patterning, where

533 Eda signaling is necessary to stabilize and refine an otherwise labile Wnt-dependent placode prepattern

534 (11,43). In the molar field, *Edar* expression in the dental lamina reaches levels pretty similar to restricted

535 expression in the signaling center, suggesting that *Edar* is actively stimulated in both cases. This

536 upregulation may rely on ActivinβA (stimulating *Edar* expression in tooth cultures, (Laurikkala et al. 2001)

537 and the Wnt pathway (which plays a central role in tooth formation, and is involved in *Edar* basal

23

538   expression in hair (11,43)). Downregulation may rely on the BMP pathway, as in hair ((11,43)). ii) We

539   show that this regulation still occurs in the *Edar* mutant, a major difference with hair, for which the

540   regulation does not occur and uniform basal levels of *Edar* expression are maintained in the absence of

541   Eda signaling (11). Self-activation of the pathway thus plays a more minor role, if any, in teeth.

542   We believe that these differences on *Edar* regulation may reflect differences in the balance of the different

543   processes participating in hair and tooth formation. For the formation of hair placodes, Turing-like

544   mechanisms establish a noisy pre-pattern, with local sources of FGF signaling. Mesenchyme condensation

545   towards these sources then refines and reinforces the pattern (Glover 2017). The mesenchyme is also able

546   of autonomous self-organisation, but this is masked by the pre-pattern imposed by the epithelium.

547   The formation of tooth signaling centers seems to rely on a different equilibrium between the two tissues.

548   The formation of a PEK is highly dependent on mesenchyme condensation, as seen in bud-arrested tooth

549   germs where condensation fails (29). Modeling the gene network of epithelium-mesenchyme interactions

550   in teeth also lead to the suggestion that the two tissues work in concert, rather than one dominating the

551   other (O'Connell *et al.,* 2012). These intrinsic differences may explain why *Edar* loss of function abolishes

552   pattern formation in the epithelium-dominated context of hair formation, but only results in spatio-temporal

553   modifications, in the more balanced context of tooth formation.

554

555   **A model for sequential patterning of signaling centers in the dental epithelium**

556   In this study, we assume that the complex spatio-temporal changes in *Edar* expression highlight waves of

557   activation in the dental epithelium. Each of these waves resumes with the patterning of a signaling center

558   and they are reiterated upon posterior growth of the dental lamina. We note that this growth zone could be

559   the *Sox2* positive region shown in (23). We built a reaction-diffusion mathematical model to describe this

560   behavior. In this macroscopic model, molecules are treated as a continuum, and set on a 1-dimensional

561   space to model the antero-posterior dimension of molar row formation. We also chose to consider only 2

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562 types of Turing in-phase molecules, corresponding to an activator and an inhibitor. This is of course a high

563 level of abstraction. Tooth genetics has revealed many molecules from the epithelium or the mesenchyme

564 that could participate in the activation-inhibition mechanisms (with both in-phase and out-of-phase

565 patterns), but it was not our purpose here to identify these molecules. Instead, our modeling effort aimed

566 at providing a theoretical framework for sequential tooth formation. Moreover, although our model

567 explicitly aims to describe activation in the epithelium (*Edar* dynamics), this does not mean that the

568 activator-inhibitor couple in our model should be seen as an abstraction for Turing reactions in the

569 epithelium only. We do not rule out that our model could synthesize the activation-inhibition reactions

570 arising from epithelia-mesenchymal interactions and giving rise to the Turing pattern.

571 We focused on qualitative insights and assessed robustness of pattern formation and developmental

572 palimpsest in our model. We found a suitable model parametrization, and tested its sensitivity with respect

573 to patterning (see Supp Mat for details). Although the results are generally robust enough to moderate

574 parameter changes (10%-50%), it is interesting that the developmental palimpsest can be abolished in

575 many ways, changing auto-inhibition but also temporal dynamics and synchronicity between events. This

576 is consistent with the marked tendency of molar row development towards supplementary molar

577 formation: it can happen in mutants from many different pathways, and moreover it often occurs without

578 major changes in other aspects of tooth development.

579 Our model explicitly assumes that the mesenchyme is responsible for periodic activation priming the newly

580 grown epithelium. This dependence is consistent with a body of evidence showing that mesenchyme

581 activity is necessary for the induction of primary enamel knot formation and sequential tooth formation

582 (61,28,27). We also know that mesenchyme activity depends on the msx1-Bmp4 feedback loop (62–64),

583 which is itself dependent on a mechanical signal provided by mesenchyme condensation (Mammoto et al.

584)(29). When this loop is defective, sequential tooth formation can stop at different stages from no tooth

585 forming, only one, or only two instead of three (65,63). It can also simply stall until adequate levels of

25

586   Bmp4 signaling are reached, as seen in the barx1 mutant (66). The mesenchymal Bmp4 signal is part of a

587   Wnt-Bmp regulatory network whose integration drives signaling center formation (28). It is also known

588   that the mesenchyme produces ActivinβA, a potent inducer of both tooth formation (Kavanagh et al. 2007)

589   and *Edar* expression. In the absence of further knowledge about how the mesenchyme could prime the

590   waves of activation observed in the epithelium, we introduced in our model an extrinsic component

591   representing the interaction with the mesenchyme, and chose a parsimonious way to provide it an

592   oscillatory behavior. For this, we assumed that the mesenchyme activity is stimulated by the activator and

593   feedbacks on it in the newly grown area. Below a certain threshold, it will act to increase the concentration

594   of the activator. Above a certain threshold, it will act to decrease the concentration of the activator.

595   Another interesting feature of our model is that inhibition from the Turing spot locks the bistable system of

596   the newly grown epithelium in the "no activation" state. This means that, in the absence of a wave of

597   activation triggered by the mesenchyme, sequential addition will stop, in contrast with a standard Turing

598   system in a growing field. This is consistent with mutants in the bmp4-msx1 axis where sequential addition

599   resumes after M1 or M2 formation. Experimental approaches will be needed to determine the mechanisms

600   enabling periodicity in our system, and to implement this in the model. Further investigation within our

601   modeling framework should involve two separated compartments for epithelium and mesenchyme. It is

602   expected that regulation feedbacks and delayed growth can trigger intrinsic oscillations if the mesenchyme

603   can escape the "locked" inactivated state under long-range inhibition.

604

605   Our model shares some similarities with models of somitogenesis. First of all, almost all somitogenesis

606   models include a clock driving gene expression oscillations, forming traveling waves moving through the

607   tissue (e.g. (14)). Even cells isolated from the presomitic mesoderm exhibit oscillations (67). However,

608   whether such a *bona fide* molecular oscillator will be found in the tooth system remains an open question.

26

609   We note that tissue-scale oscillations have been observed in limbs, whose development share similarities

610   with that of epithelial appendages including teeth (68). We also envision other possibilities relying on tissue

611   properties rather than cell properties, for example emerging from the cross-talk between the epithelium and

612   the mesenchyme (as suggested above).

613   Second, in the long-prevailing models of somitogenesis, the clock is combined with a gradient of Fgf/Wnt

614   signaling, that maintains the oscillations in the posterior part and determines the position where the

615   traveling wave is frozen into a stationary pattern, which will define somite boundaries (69). Our model

616   does not comprise such positional information. In a more recent model of somitogenesis, traveling wave

617   and pattern formation are produced by a Turing pair with a non-diffusing activator and a diffusing inhibitor

618   (Cotterel et al. 2015). Pattern formation arises when the traveling wave breaks next to the previously formed

619   stripe (that acts as a stable source of inhibitor), and local interactions in this region promote activator

620   increase to form a new Turing stripe in the vicinity of the previous stripe. This model shares an obvious

621   similarity with our model: a Turing pair exhibits different behaviors (oscillatory with traveling wave /

622   Turing in the Cotterel model versus bistable with traveling wave/Turing in our model) along the antero-

623   posterior axis. However, the switch between the two behaviors arises as a local emergent property next to

624   previously formed stripes in the Cotterel model, whereas it is explicitly introduced in our model as a result

625   of maturation. Moreover, in our model the oscillations are provided as an independent term, materializing

626   mesenchyme function. We acknowledge that the Cotterel model might apply to the tooth system, and it

627   will be interesting to test if the palimpsest can be obtained with such a model.

628   Our study also shares superficial similarities with another system showing sequential patterning: feather

629   patterning. In this system, a priming wave of activation is observed in the epithelium, giving rise to a stripe

630   in the chick embryo's back, which is then broken into a spot pattern giving rise to individual feathers.

631   Pattern formation, in the model by Painter et al., relies on chemotaxy rather than reaction-diffusion (21):

632   moderate cell aggregation drives stripe formation in the primed epithelium through a FGF-dependent

27

633   positive feedback, and strong local aggregation introduces a BMP-dependent negative feedback that

634   contributes to break the stripe into spots. The behaviors of the two systems are similar: the broad Edar

635   expression could be compared to the priming wave/first stripe, and the formation of the signaling centers

636   to the breaking of the stripe into spots. These models also converge conceptually. Stripe formation in the

637   feather model, and Edar activation wave in the tooth model, mainly rely on positive feedback. Spot

638   formation and signaling center formation both rely on the introduction of a sharper negative feedback. We

639   take this as an indication that this sequence of activation might be a general property of epithelial

640   appendages (feathers, hair, teeth), that can be captured by very different, non-exhaustive models. We also

641   want to stress that our model is meant to recapitulate activation/long-range inhibition mechanisms, rather

642   than specifically reaction-diffusion mechanisms, and we do not exclude that the biological mechanisms it

643   captures are based on chemotaxy, as in the Painter model.

644

645   **Making and erasing patterns: a developmental palimpsest characterizes first molar formation**

646   Lastly, we would like to emphasize how the current model successfully recapitulates a number of counter-

647   intuitive behaviors of the system and inform us on the possible underlying mechanisms.

648   Previous studies had already revealed several complex behaviors in the growing dental epithelium: i) the

649   transient patterns of MS and R2 signaling centers, supposedly vestiges of premolar signaling centers; ii)

650   the rescue of an abortive tooth germ R2, in a large number of genetic conditions; iii) the transient co-

651   existence of R2 and early M1 signaling center followed by their fusion in a large signaling center in the

652   lower jaw.

653   The present data and our simple model suggest that these complex behaviors are the fruit of rather simple

654   but highly dynamic interactions in the growing tooth field.

655   As viewed from Edar expression, the pattern constituted by MS, and later R2 signaling centers is erased to

656   give raise to a second wave of patterning, materialized by a broad Edar expression in the dental epithelium

28

657   at respectively 12.5-13.0 dpc and 13.5-14.0 dpc. This was recapitulated in the model by enabling the

658   bistable domain to form a traveling wave, that can destabilizes a previously formed signaling center, if

659   inhibition in the later is not too strong. Aside from recapitulating Edar expression, the travelling wave has

660   more profound implications. Indeed, it implies a first paradigm shift that vestigial buds are not committed

661   to abort as usually thought (for example due to their proximity with the diastema thought to serve as a

662   source of inhibitors (70,71), or through the expression of specific molecules (72)), but rather (or on top of

663   that) that they are actively competed by the next round of activation as the dental epithelium grows. Such

664   a balance explains why the anterior part of the molar row is very sensitive to genetic perturbations (with

665   many genetic conditions exhibiting a supplementary tooth there) and environmental perturbations (i.e.

666   tooth culture in this study). It also explains why even conditions that produce a more inhibitory context

667   than the wild type can produce such supplementary tooth. Indeed, our model predicts that if inhibition is

668   increased (e.g. auto-inhibition is decreased), like it is commonly assumed in the Eda pathway mutants, then

669   the Turing pattern remains (with a slightly longer wavelength), but the traveling wave is almost

670   immediately suppressed. This is exactly what we document in Edar^{DlJ} mutants for the R2 signaling center:

671   it forms more posteriorly, and we see no traveling wave that would erase it. Rather, it persists to form a

672   tooth bud. Our cultivation of anterior parts of the molar field, corresponding to R2 signaling center, also

673   show that it has the potential to fully form a tooth, but is actively competed by the M1 signaling center in

674   the wild type situation. Consistent with our results, Li *et al.* reported that FGF8 application could rescue

675   tooth germ development in the mouse diastema only when it was separated from the molar and incisor buds

676   (73). In conclusion, our results extend the prevailing model (that of Kavanagh et al.), where inhibition

677   between forming teeth is unidirectional (from M1 to M2, to M3), by showing that inhibition can be

678   bidirectional and subtly dependent on the temporal dynamics of the system.

679   In the wild type, following broad *Edar* activation at 13.5-14.0, a new pattern of *Edar* restriction forms that

680   is markedly different between the lower and upper jaw. In the lower jaw, independent R2 signaling center

29

681 and early M1 signaling center are transiently seen (very transiently with *Edar* expression, for a longer time

682 with *Shh* expression or TOPGAL activity) but they are rapidly included in a single elongated signaling

683 center. In the upper jaw, *Edar* restricts to R2 signaling center and M1 signaling center and remains as such.

684 Therefore, the palimpsest is observed in both jaws, enabling the co-occurrence of two signaling centers,

685 but only in the lower jaw does some additional mechanism enable their fusion into a large signaling center.

686 Here, we introduced chemotaxy to the model, because chemotaxy has been evidenced in hair placode

687 formation, both at the level of the epithelium (45,46) and the mesenchyme (10,21). This was sufficient to

688 recapitulate a number of interesting features: 1) chemotaxy changes the reaction-diffusion so that the

689 system first makes two peaks that later fuse into a single, larger peak: this is reminiscent of the large M1

690 signaling center 2) this behavior is sensitive to the distance between the initial peaks, and a 15% increase

691 was sufficient to impede fusion. The measured 30% difference between the R2-M1 distance in the lower

692 jaw, where fusion occurs, and the R2-M1 distance in the upper jaw, where fusion does not occur, may thus

693 be sufficient to explain difference in fate in the two jaws. 3) finally, reducing chemotaxy was sufficient to

694 impede fusion. This may explain why in our culture system, inhibition of Edar activity impedes fusion

695 although the distance between R2 and M1 does not seem drastically changed.

696 Interestingly, we show that chemotaxy plays an ambivalent role in our model. Depending on the conditions,

697 it acts in favor or against the Turing pattern, or it is relatively neutral. We propose that this ambivalent role

698 contributes to explain the versatility of this biological system in regard to genetical and environmental (e.g.

699 culture) perturbations.

700

701

702 **Conclusion**

703 An important lesson from the tooth system is that patterning events may be less straightforward

704 than usually thought, and patterns may be dynamically drawn and erased or refined during

30

705   embryogenesis. In other words, developmental palimpsests may be a common feature. One

706   reason for this is historical: systems are the product of evolution, and as pointed out by F. Jacob,

707   evolution proceeds as a tinkerer, not as an engineer (74): extant patterning mechanisms are

708   modified versions of ancestral mechanisms, and not purposely designed from scratch. Our study

709   is consistent with recent studies on the fine-scale temporal dynamics of gap gene patterns in

710   dipterans (e.g. a progressive anterior shift of the gap genes pattern). As shown here for the *Edar*

711   mutant, incorporating this dynamics into models provided a better explanation for mutant

712   phenotypes (8,75,76). Moreover, this curious dynamics is also likely the vestige of an ancestral

713   mode of segmentation (75,77). In summary, we believe these two systems illustrate that temporal

714   dynamics of developmental systems needs to be studied, and moreover to be studied in the light

715   of evolution, to fully explain how the system reacts to perturbations. Indeed, embryonic patterns

716   can be highly dynamic and thus dynamics can be essential to the outcome of the patterning

717   process.

718

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720

31

Materials and Methods

Mouse breeding and embryo harvesting and staging.

All animal experimentations were conducted under animal care procedures in accordance with the guidelines set by the European Community Council Directives. Experimental procedures were approved by an official ethical committee (CECCAPP, Lyon; # ENS-2009-027 et # ENS-2012-046).

The CD1 mice were purchased from the Charles River (Germany). Other mice have been bred at the PBES (Lyon). TOPGAL mice (Tg(Fos-lacZ)34Efu) carrying three LEF1/TCF1 binding sites fused to a minimal *c-fos* promoter driving *lacZ* expression were backcrossed against CD1 mice for 10 generations {DasGupta, 1999 #6}. TOPGAL positive mice were screened by standard lacZ staining performed on the first phalange cut from PN4-PN7 newborns. The *Edar*[dl-J] mice (FVB background) were obtained from Paul Overbeek. They carry a G to A transition mutation causing a glutamate to lysine substitution in the death domain of the Edar protein (E379K, Headon and Overbeek, 1999). The strain was maintained by crossing heterozygotes with homozygotes, and wild type and *Edar*[dl-J/dl-J] mice used in experiments were derived from this same stock. The Eda[Tabby] mice carry a X-linked null mutation in the *Eda* locus with a deletion of the first exon (Probst 2008). The colony was established by inbreeding from a mating pair (B6CBACa Aw-J/A-Eda[Ta]/J-XO female and B6CBACa Aw-J/A male) obtained by the Jackson Laboratory (Bar Harbor, Maine).

In order to harvest embryos every 12h hours of development, mice were kept under two different day-night light cycles. Mice were mated overnight and vaginal plugs were detected in the next morning, noon being indicated as the embryonic day (ED) 0.5. Pregnant mice were killed by cervical dislocation and embryos were harvested and weighted as described earlier {Peterka, 2002 #30; Prochazka et al 2010}.

For tooth culture experiments, CD1 females were crossed with males carrying the fusion protein Shh-EGFP

32

743   (Enhanced Green Fluorescent Protein) and Cre recombinase from the endogenous *Shh* locus (B6.Cg-

744   *Shh$^{tm1(EGFP/cre)Cjt}$*/J) what enabled determination of *Shh* expression using fluorescence. The breeding pairs

745   B6.Cg-*Shh$^{tm1(EGFP/cre)Cjt}$*/J were purchased from the Jackson Laboratory (Maine, USA). The mice were

746   genotyped using the Jackson Laboratory's protocols. All used animals were fed and watered ad libitum.

747   Housing of animals and experiments were carried out in strict accordance with the national and

748   international guidelines (ID 39/2009) and under supervision of the Professional committee for guarantee

749   of good life-conditions of experimental animals at the Institute of Experimental Medicine, the Czech

750   Academy of Sciences, Prague, Czech Republic and approved by the Expert Committee at the Academy of

751   Sciences of the Czech Republic (permit number: 81/2017).

752   **Mandible epithelium dissociations.** Mandibles and maxilla were dissected in Hank's medium and treated

753   with Dispase II (Roche) 10mg/ml at 37°C for 1 to 2h20 depending on embryonic stage. Epithelium was

754   carefully pealed and fixed in PFA 4%.

755   **Whole mount In situ hybridization (WISH) and X-gal staining.** Embryonic mandibles, maxilla or

756   dissociated epithelia were fixed in 4% PFA solution over night at 4˚C and *In situ* hybridization was done

757   according to a standard protocol. DIG RNA probes were transcribed *in vitro* from plasmids described

758   elsewhere: *Shh* {Echelard, 1993 #1}, *Edar* {Laurikkala, 2001 #75}. TOPGAL embryonic mandibles or

759   dissociated epithelia were fixed in 4% PFA for 15 minutes only and stained with X-gal according to a

760   standard protocol. The samples were documented on a Zeiss LUMAR stereomicroscope with a CCD

761   CoolSNAP camera (PLATIM, IFR128, Lyon) or on a LEICA MFA205 stereomicroscope with a DFC450

762   camera (IGFL, Lyon).

763   **Organotypic culture and treatments.** The lower molar region of 13.0 embryos were dissected and

764   cultured according to methods described in Kavanagh et al. 2009. Following a period of 2 hours of

765   recovery, the medium was changed for a new medium supplemented with 5ug/ml Eda interfering antibody

766   (ectoD3; (78)). Tooth culture was stopped at 40h and epithelium were dissociated for 15-30 minutes

33

767   Dispase II (Roche) 10mg/ml at 37°C.

768

769   **In vitro cultures of anterior and posterior parts of M1 tooth primordium**. M1 tooth germs of

770   *ShhEGFP$^+$* mouse embryos at 14.3 dpc were dissected from embryonic lower jaw and cut to anterior and

771   posterior part. Both parts were cultured separately on PET track-etched membrane. Contralateral intact M1

772   dissected tooth germs from the same specimen were used as control. Cultures were photographed using

773   inverted fluorescent microscope Leica AF6000 (Leica Microsystems GmbH, Germany) daily from day of

774   dissection to day 6 of culture.

775

776   **Mathematical modeling**

777   The model is described in supplementary material 1.

778

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779

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785

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786

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989

Supporting information

990

991

992 **Supplementary material 1 (.pdf):** a detailed description of the model together with simulations under

993   different parameter ranges.

994

995 **Supplementary material 2:** a movie (.gif) of the simulation shown in figure 2. The evolution of activator

996   concentration (red), inhibitor concentration (blue) and domain maturation (green) are shown as the
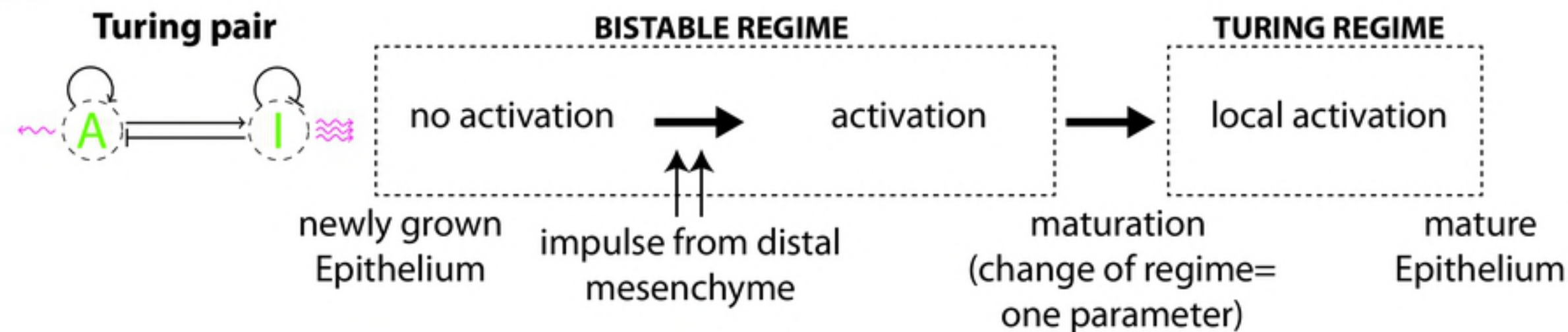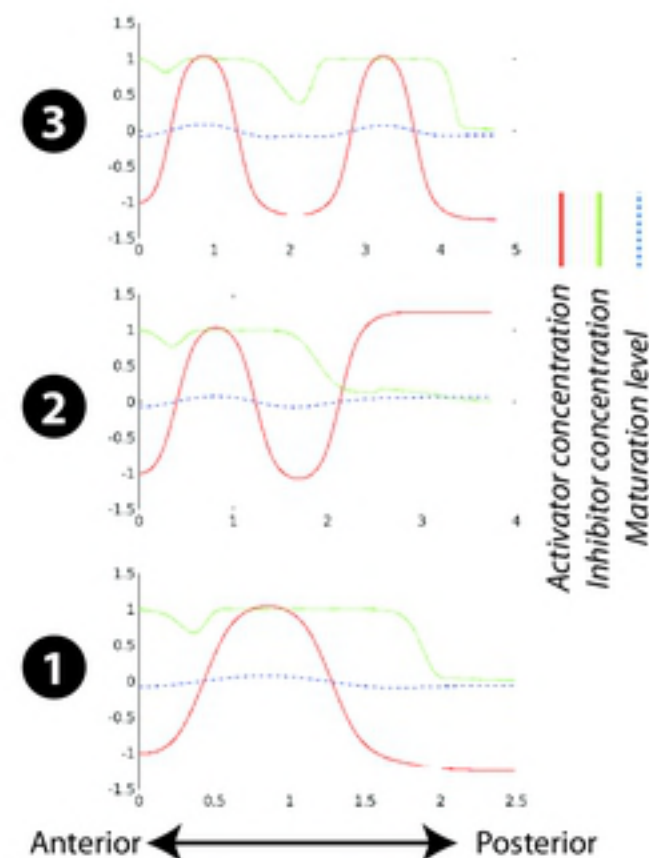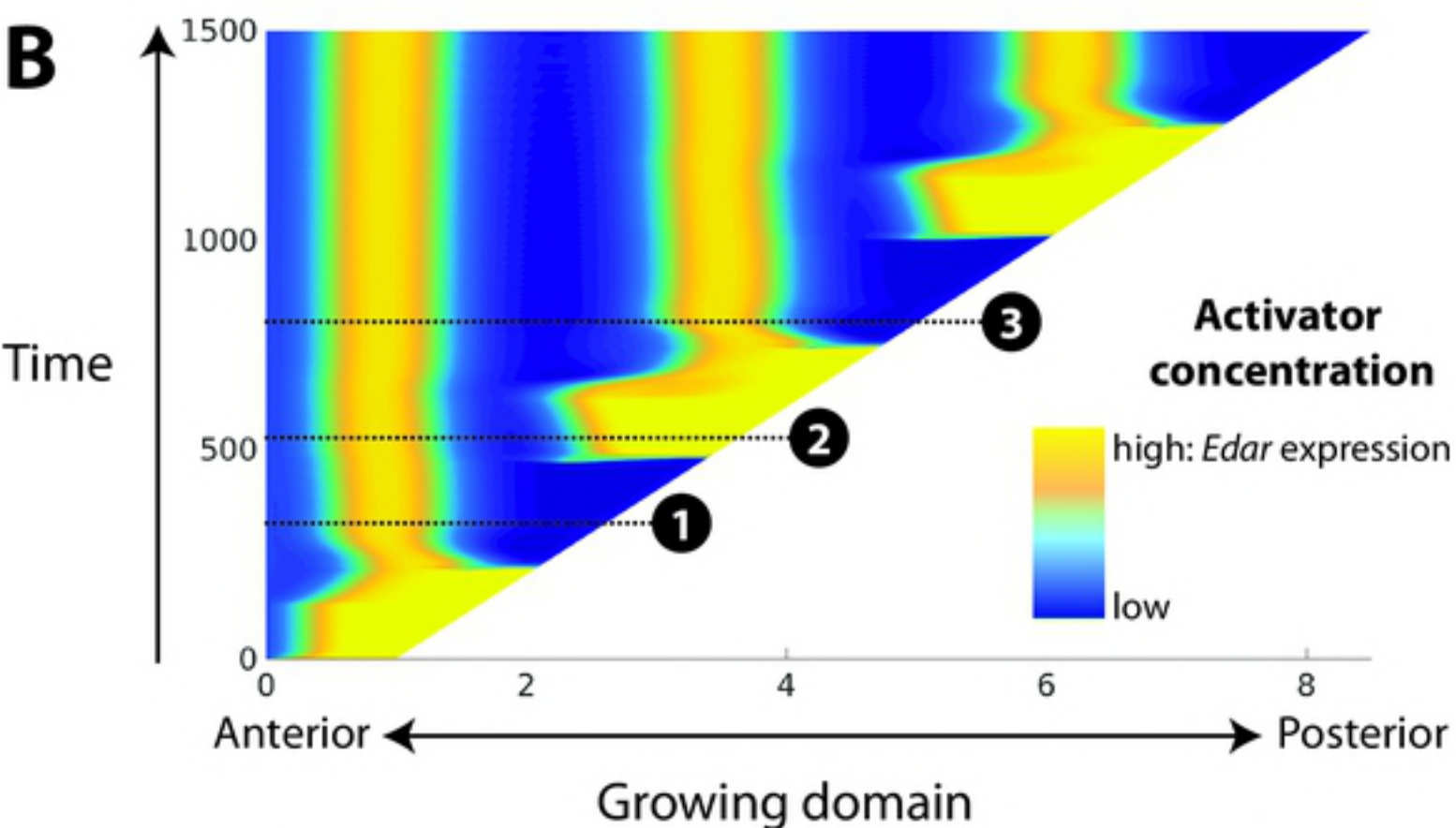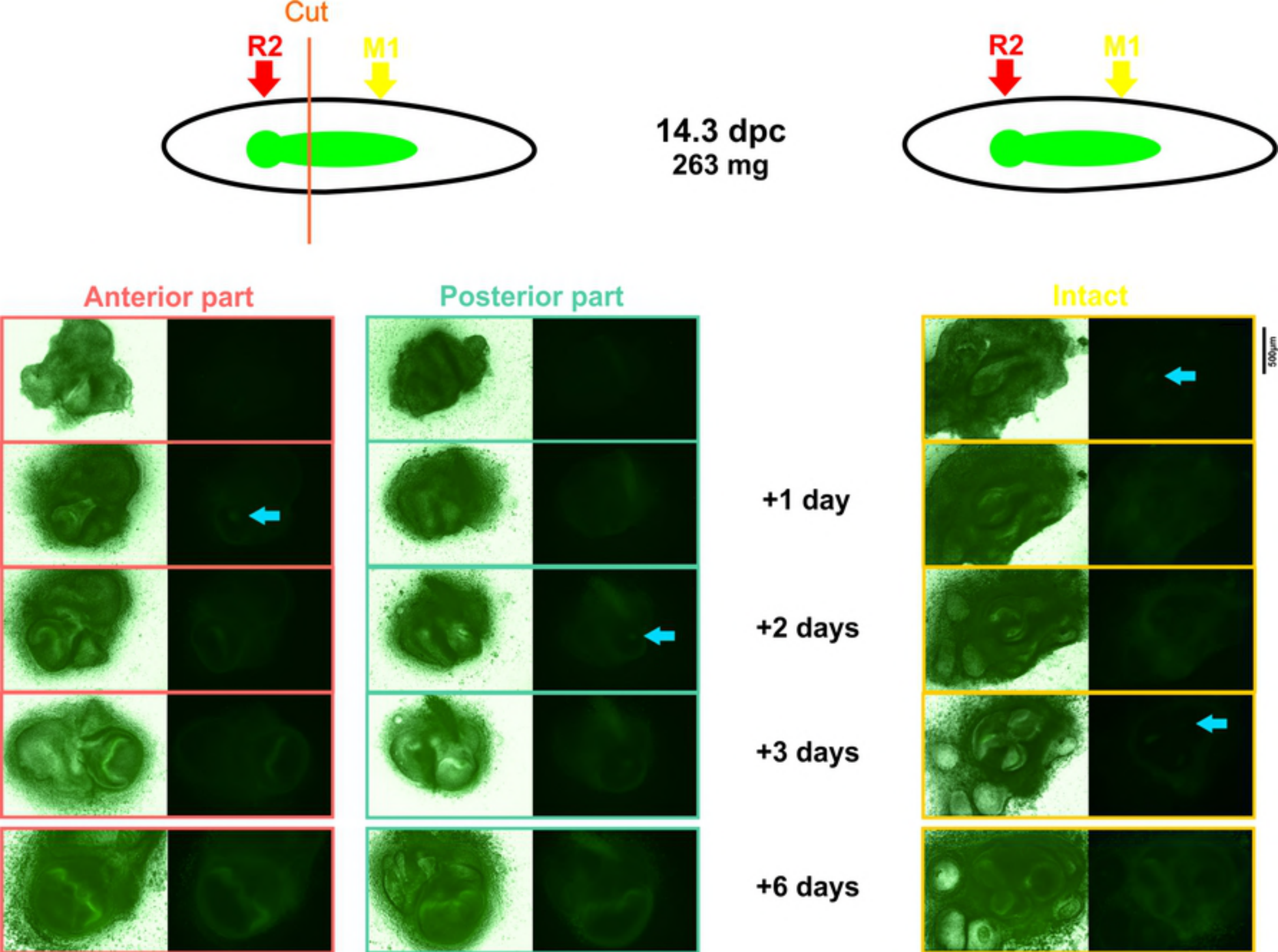
997   domain grows.

998

999 **Supplementary material 3:** a movie (.gif) of the simulation shown in figure 3. The evolution of activator

1000   concentration (red), inhibitor concentration (blue) and domain maturation (green) are shown as the

1001   domain grows.

1002

1003 **Supplementary material 4:** a movie (.gif) of the simulation shown in figure 4. The evolution of activator

1004   concentration (red), inhibitor concentration (blue) and domain maturation (green) are shown as the

1005   domain grows.

1006

A

Turing pair

A ⊣ I

BISTABLE REGIME

no activation → activation

newly grown
Epithelium

impulse from distal
mesenchyme

TURING REGIME

local activation

maturation
(change of regime=
one parameter)

mature
Epithelium

B

Time

1500

1000

500

0

**Activator
concentration**

high: *Edar* expression

low

0   2   4   6   8

Anterior ← → Posterior

Growing domain

① ② ③

Activator concentration
Inhibitor concentration
Maturation level

Anterior ← → Posterior
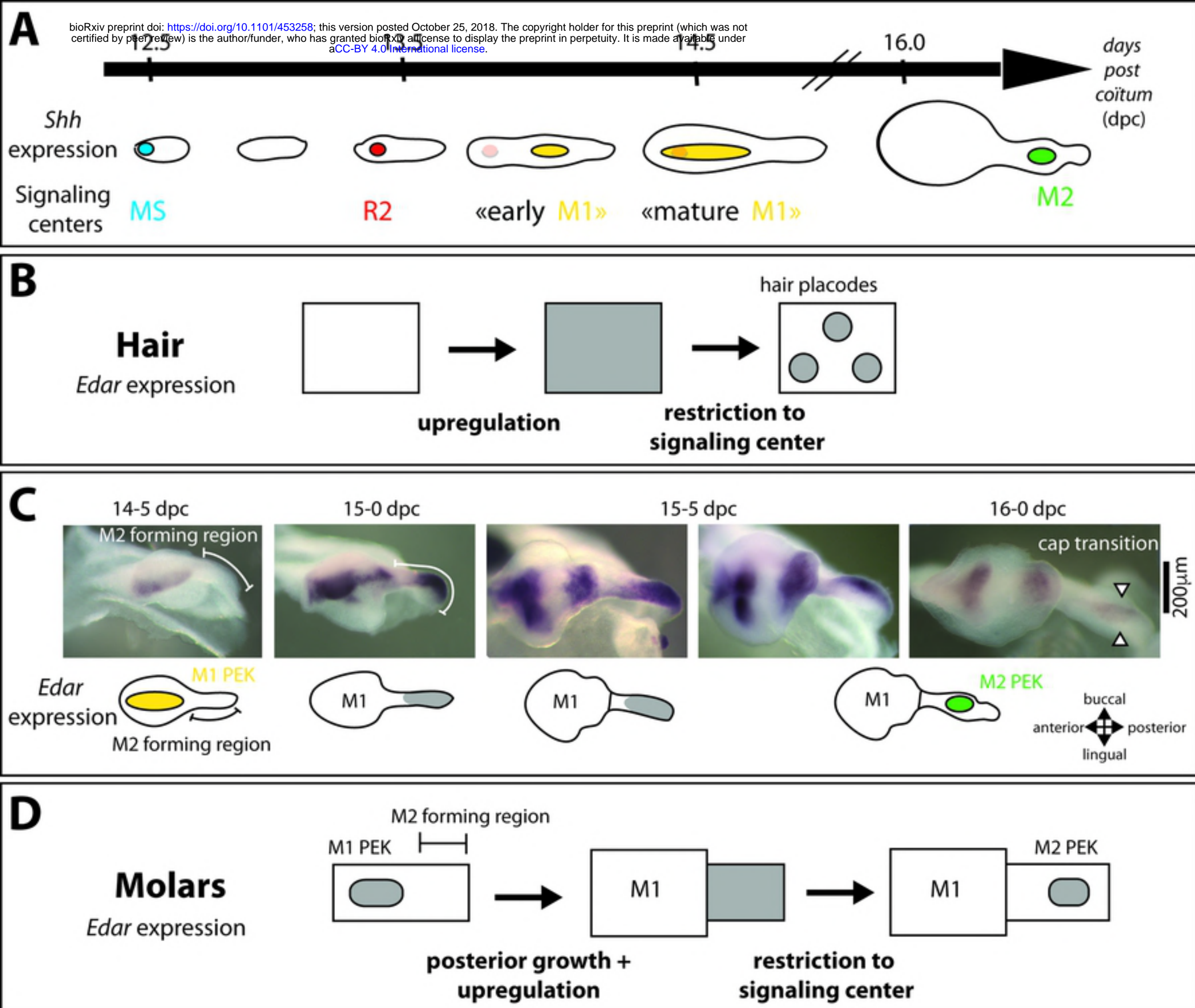
Figure 2

Figure 5

Figure 1
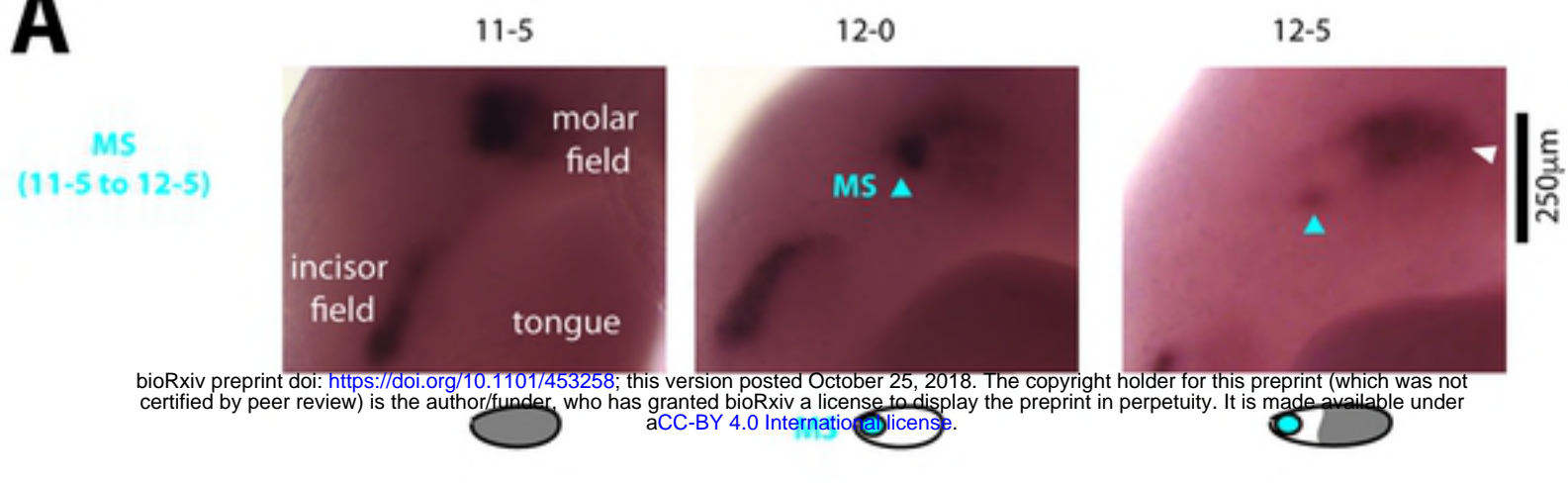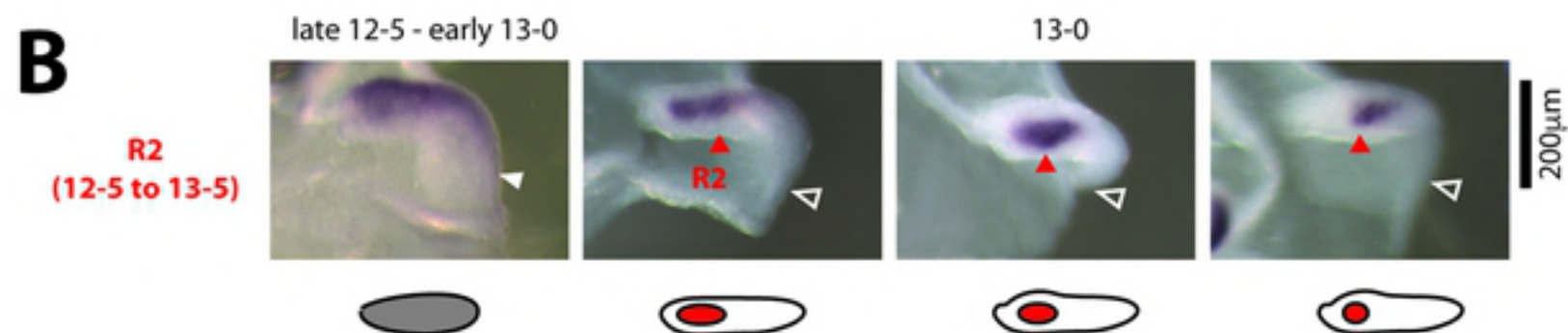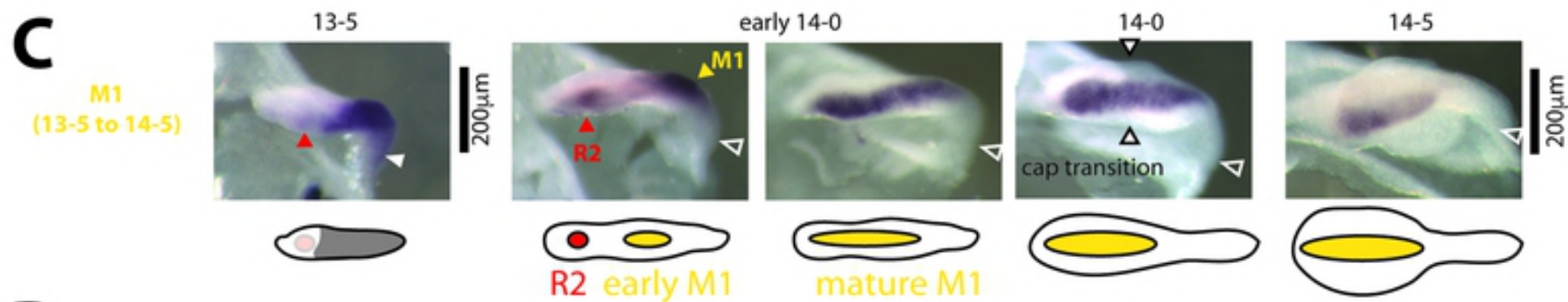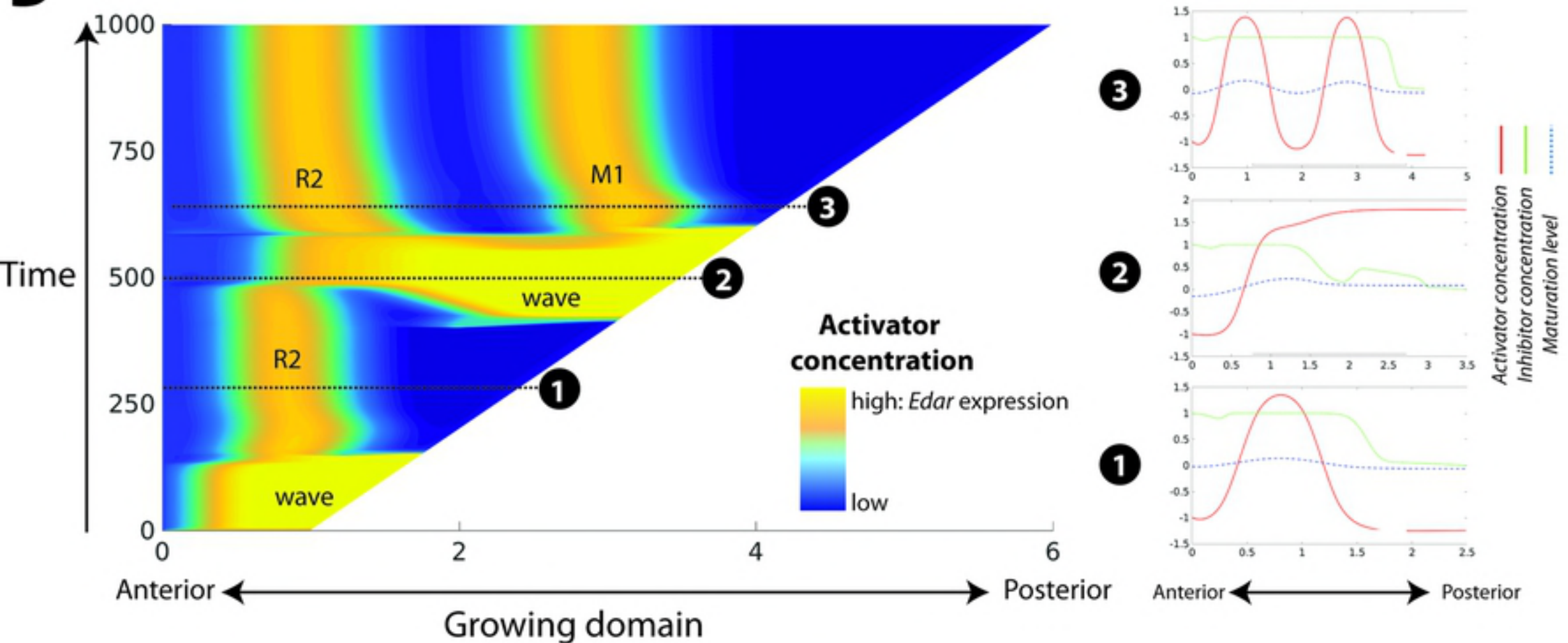
Figure 3

Figure 4

Figure 6