# The *shh* limb enhancer is activated in patterned limb regeneration but not in hypomorphic limb regeneration in *Xenopus laevis*

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Summary statement: We visualized the activity of a limb-specific enhancer of *shh* in the regenerating limb of an amphibian, *Xenopus*: it correlates with the capacity for regeneration of a well-patterned limb.

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## ABSTRACT

*Xenopus* young tadpoles regenerate a limb with the anteroposterior (AP) pattern, but metamorphosed froglets regenerate a hypomorphic limb after amputation. The key gene for AP patterning, *shh*, is expressed in a regenerating limb of the tadpole but not in that of the froglet. Genomic DNA in the *shh* limb-specific enhancer, MFCS1 (ZRS), is hypermethylated in froglets but hypomethylated in tadpoles: *shh* expression may be controlled by epigenetic regulation of MFCS1. Is MFCS1 specifically activated for regenerating the AP-patterned limb? We generated transgenic *Xenopus laevis* lines that visualize the MFCS1 enhancer activity with a GFP reporter. The transgenic tadpoles showed GFP expression in *hoxd13*- and *shh*-expressing domains of developing and regenerating limbs, whereas the froglets showed no GFP expression in the regenerating limbs despite having *hoxd13* expression. Genome sequence analysis and co-transfection assays using cultured cells revealed that Hoxd13 can activate *Xenopus* MFCS1. These results suggest that MFCS1 activation correlates with regeneration of AP-patterned limbs and that re-activation of epigenetically inactivated MFCS1 would be crucial to confer the ability to non-regenerative animals for regenerating a properly patterned limb.

# INTRODUCTION

Among tetrapods, only urodele amphibians (newts and salamanders) can regenerate an amputated limb completely throughout their life cycles (Han et al., 2005; Joven et al., 2019; Otsuki and Tanaka, 2022; Agata and Inoue, 2012). In the anuran amphibian *Xenopus laevis*, a tadpole can completely regenerate its limbs prior to the onset of metamorphosis, but it loses regenerative capacity gradually as metamorphosis proceeds (Dent, 1962; Muneoka et al., 1986). The froglet, a young post-metamorphosis adult, regenerates only an unbranched cartilaginous spike-like structure after limb amputation (Dent, 1962; reviewed in Suzuki et al., 2006): the spike lacks the pattern formation along the anteroposterior (AP) axis. In the limb formation process, *sonic hedgehog* (*shh*) is expressed in the posterior part of the limb bud and serves as the key gene for the AP axis formation (Riddle et al., 1993; Harfe et al., 2004). *shh* is also expressed in the posterior part of a regenerating limb (blastema) in urodeles (Imokawa and Yoshizato, 1997; Torok et al., 1999). In *Xenopu*s, *shh* is expressed in the posterior part of the limb blastema of a tadpole (Endo et al., 1997) but is not expressed in the blastema of a froglet limb (Endo et al., 2000; Yakushiji et al., 2007). Treatment with a Hedgehog inhibitor results in hypomorphic limb regeneration in both a *Xenopus* tadpole and an axolotl (Roy and Gardiner, 2002; Satoh et al., 2006). These results suggest that *shh* re-expression correlates with the limb regenerative capacity: whether a limb regenerates with the AP pattern or not.

Genetic studies in mice have revealed that a long-range cis-acting enhancer called MFCS1 (mammal-fish-conserved-sequence 1), also called ZRS (ZPA regulating sequence) or Rr29, regulates limb-specific *Shh* expression (Lettice et al., 2003; Sagai et al., 2004). In transgenic (Tg) mouse embryos, MFCS1 with a minimal promoter drives *lacZ* expression in the posterior part of a limb bud (Lettice et al., 2003). Depletion of a large part of this sequence in a mouse results in limb-specific loss of *shh* expression and in loss of the AP polarity of a limb (Sagai et al., 2005). The activity of MFCS1 is positively regulated by Hoxd13 (5' Hoxd) and ETS transcription factors (ETS1 and GABPα), downstream factors of FGF signaling in a mouse limb bud that directly bind to MFCS1 (Capellini et al., 2006; Lettice et al., 2012). In amphibian limbs, the methylation level of genomic DNA in MFCS1 is relatively high in the limbs (both intact and

120 regenerating limbs) of a *Xenopus* froglet, while the methylation level is low to medium
121 in the limbs of a *Xenopus* tadpole, an axolotl and a Japanese newt (Yakushiji et al., 2007).
122 These findings strongly suggest that epigenetic inactivation of MFCS1 leads to the
123 shutdown of *shh* expression that results in hypomorhic limb regeneration without the AP
124 pattern in a froglet. The importance of MFCS1 for limb regeneration is further supported
125 by the results of partial deletion of the MFCS1 sequence by CRISPR/Cas9 in an Iberian
126 ribbed newt: the partial deletion resulted in reduction in *shh* expression and reduction in
127 the number of digits in limb regeneration (Suzuki et al., 2018). However, the spatio-
128 temporal activation pattern of MFCS1 has not been visualized in regenerating limbs of
129 amphibians. Thus, it is still unknown whether MFCS1 is activated in regenerating limbs
130 of amphibians.

131 Here, we succeeded in visualizing and monitoring the spatio-temporal pattern of
132 MFCS1 activation in regenerating limbs as well as in developing limbs by establishing
133 Tg *Xenopus laevis* lines carrying a GFP reporter linked to MFCS1 (MFCS1-GFP).
134 Expression patterns of MFCS1-GFP were overlapped with those of *shh* both in limb
135 development and limb regeneration. MFCS1-GFP expression was specifically localized
136 in the regenerating limb of a young tadpole. The pattern of MFCS1-GFP expression
137 coincided with the expression pattern of *hoxd13* in both developing and regenerating
138 tadpole limbs. Furthermore, genome sequence analysis and luciferase assays *in vitro*
139 suggested a positive regulation of *Xenopus* MFCS1 by Hoxd13. However, despite *hoxd13*
140 expression, MFCS1-GFP expression was not found in the regenerating froglet limb. We
141 revealed that the DNA methylation level of exogenous MFCS1 of the transgene as well
142 as that of endogenous MFCS1 in the regenerating limb were higher in the froglet than in
143 the tadpole. Our results suggest that MFCS1 is activated in limb regeneration with the AP
144 pattern but is inactivated in hypomorphic limb regeneration after metamorphosis when
145 MFCS1 has a higher DNA methylation status than that in tadpole stages.
146

147 # RESULTS AND DISCUSSION

148 **MFCS1-GFP expression localizes in the posterior mesenchyme of a developing limb**
149 **bud**

150 To visualize the MFCS1 activity by the GFP reporter, we generated a transgene construct

151 containing GFP immediately downstream of a *Xenopus shh* promoter sequence
152 (Yakushiji et al., 2007) (Fig. 1A). At the same time, an approximately 3.2-kbp *Xenopus*
153 MFCS1 sequence was cloned and inserted upstream of the *shh* promoter in the transgene
154 construct (Fig. 1A). We generated transgenic *X. laevis* containing this transgene named
155 "*Xla.Tg(Xtr.shh:GFP)*$^{Tamura}$" (according to the Xenbase nomenclature guideline). For
156 simplicity and for highlighting the involvement of the limb-specific enhancer, MFCS1,
157 we hereafter refer to this Tg as MFCS1-GFP. We observed the GFP pattern in G0 founder
158 animals. Since *shh* is most prominently expressed in the posterior part of a limb bud at
159 stage 51-52 (Endo et al., 1997; Fig. 1B), we firstly focused on limb buds at stage 51-52
160 (Nieuwkoop and Faber, 1994). GFP was found in the posterior part of the limb bud in at
161 least eleven independent G0 individuals and the fluorescence gradually diminished after
162 stage 52. Thus, we considered that the pattern of MFCS1-GFP expression reflects the
163 activity of the MFCS1 enhancer. We established two independent lines by mating selected
164 GFP-expressing founder animals with wild-type frogs (Table S1). About 50% of the N1
165 individuals of line 2 as well as the N1 and N2 individuals of line 1 were GFP-positive in
166 limb buds, presumably being inherited in a Mendelian manner. Consistent with G0
167 animals, limb buds of both line 1 and line 2 at stage 51-52 showed GFP expression in the
168 posterior part (Fig. 1C, C' and Fig. S1A, A'). In the following experiments, we used GFP-
169 positive individuals of line 1 (N1 or N2) and line 2 (N1).

170 Confocal imaging revealed that GFP was localized specifically in the posterior
171 mesenchyme of the limb bud at stage 51-52 (Fig. 1D, E and Fig. S1B, C). In limb
172 development, GFP was firstly observed at stage 50 around the apical tip of a limb bud
173 (Fig. 1F, F' and Fig. S1D, D'). This observation is consistent with the initial weak *shh*
174 expression detected at stage 50 by whole mount *in situ* hybridization (Endo et al., 1997).
175 GFP expression became strongest at stages 51 and 52 and the fluorescence was more
176 posteriorly polarized by stage 52 (Fig. 1G, G', H, H' and Fig. S1E, E', F, F'). At stage 53,
177 GFP was found in the distal-posterior part of the limb bud (Fig. 1I, I' and Fig. S1G, G')
178 and the fluorescence was weaker than that at stage 52. Finally GFP expression was almost
179 undetectable at stage 54 (Fig. 1J, J' and Fig. S1H, H'). These observations were, in
180 principle, consistent with the spatio-temporal *shh* expression patterns (Endo et al., 1997),
181 while the domain of GFP expression appeared to be broader than that of *shh* expression.
182 We concluded that the MFCS1-GFP expression pattern in the Tg lines mostly visualizes

183 the activity of the endogenous MFCS1 enhancer in a developing limb bud.
184
185 **MFCS1-GFP expression predicts the AP duplication of a limb bud**
186 To further examine whether MFCS1-GFP expression correlates with limb morphogenesis
187 along the AP axis, we conducted a 180° rotation experiment on limb buds of Tg (line 1)
188 tadpoles (Fig. 2A). Cameron and Fallon (1977) found limb duplication along the AP axis
189 after rotation of the distal limb bud tips 180° on the proximo-distal axis at stage 51-53 in
190 *X. laevis*. Endo et al. (1997) repeated this experiment at stage 53 and found that ectopic
191 *shh* expression was induced in the posterior region of the graft and stump. To test the
192 correlation between MFCS1 enhancer activity and limb duplication along the AP axis,
193 we repeated this experiment and examined the GFP expression pattern (Fig. 2). Consistent
194 with the preceding results in control limb buds, in which the amputated distal tip of each
195 limb bud was returned to the stump in the original orientation, showing only faint ectopic
196 *shh* expression (Endo et al., 1997), the majority of the control limb buds (ten of 12
197 samples) without rotation did not show GFP expression and did not result in limb
198 duplication (Fig. 2B, C). In contrast, the majority of 180° rotated limb buds (eight of 11
199 samples) showed ectopic induction of GFP expression in the posterior part of the distal
200 limb bud tip (graft) and finally formed duplicated limbs along the AP axis (the number
201 of digits in a limb being more than 5) (Fig. 2D-G). These results (summarized in Table
202 S2) suggest that ectopic induction of MFCS1 activity is closely related to ectopic *shh*
203 expression and subsequent limb duplication along the AP axis, further suggesting that
204 MFCS1-GFP expression faithfully reflects the endogenous *shh* enhancer activity in a
205 limb.
206
207 **MFCS1 is activated in a blastema that regenerates a limb with the AP pattern.**
208 To examine whether MFCS1 is activated in limb regeneration with the AP pattern, we
209 amputated a stage 54 limb bud at the level of the presumptive ankle: this amputation
210 results in almost complete limb regeneration along the AP axis (Muneoka et al., 1986).
211 By this stage, *shh* expression and MFCS1-GFP expression in an intact limb bud have
212 become almost undetectable by *in situ* hybridization and observation under a fluorescence
213 stereomicroscope, respectively (Fig. 1 and Fig. S1) (Endo et al., 1997). At 4 days post
214 amputation (dpa), GFP was observed broadly in the regenerating blastema (Fig. 3R and

215 Fig. S2B-B''), indicating that MFCS1 is re-activated in the blastema that regenerates a
216 well-patterned limb.
217 MFCS1-GFP expression was distributed throughout the blastema and did not
218 appear to be localized posteriorly. It became stronger at 6 dpa (Fig. S2C-C'') and the
219 expression gradually diminished after the blastema showed a pallet shape and became
220 undetectable at 10 dpa (Fig. 3S, T and Fig. S2). *shh* expression was detected in the
221 posterior part of a limb bud at 4 dpa by *in situ* hybridization (Fig. 3E). Based on these
222 results, we judged that MFCS1-GFP expression is distributed broadly in regenerating
223 blastemas and that the temporal expression pattern of MFCS1-GFP coincidences with
224 that of *shh* expression, while the spatial MFCS1-GFP expression pattern is wider than the
225 *shh*-expressing domain. MFCS1-GFP signal in the regenerating blastema of a young
226 tadpole suggests that MFCS1 is re-activated in a regenerating limb with the AP pattern
227 formation when *shh* is expressed: *shh* expression in the regenerating limb as well as in
228 the developing limb seems to be regulated by the common enhancer, MFCS1.
229
230 **MFCS1-GFP expression pattern correlates with the pattern of *hoxd13* expression in**
231 **a tadpole**
232 Hoxd13 and FGF downstream factors, ETS transcription factors, positively regulate the
233 MFCS1 enhancer in mice (Capellini et al., 2006; Lettice et al., 2012). We examined *dusp6*
234 (*mkp3*) expression and *hoxd13* expression as indicators of positive upstream regulators
235 of MFCS1. *dusp6* expression is induced by FGF signaling (Eblaghie et al., 2003;
236 Kawakami et al., 2003) and it seems to reflect the activity of downstream factors of FGF
237 signaling, especially ETS transcription factors (Ekerot et al., 2008). We chose a stage 51
238 limb bud and a 4 dpa blastema of a tadpole in which the limb bud was amputated at the
239 presumptive ankle level at stage 54 as representatives of developing and regenerating
240 limbs, respectively: both initiate prominent *shh* expression in the posterior side (Fig. 3E,
241 I). In a developing limb bud, *dusp6* is expressed broadly in a limb bud and strong
242 expression is seen especially in the apical mesenchyme (Fig. 3K). *hoxd13* is expressed in
243 the posterior mesenchyme of a limb bud (Fig. 3L) and its expression domain is wider than
244 the *shh*-expressing domain (Fig. 3I). The posterior mesenchymal region of a limb bud is
245 positive for both *dusp6* and *hoxd13* and the double positive region seems to coincide with
246 the fluorescence of MFCS1-GFP (Fig. 1C', D, G') and *gfp* transcript (Fig. 3J) patterns.

247 The *gfp* transcript was not detected in line 2 limb bud samples (n=0/6) but was detected
248 in line 1 limb bud samples (n=10/10) by *in situ* hybridization, suggesting that line 1 has
249 stronger transgene expression than that of line 2. The coincidence between upstream
250 regulators and MFCS1-GFP expression seems to be clearer in a regenerating limb: *dusp6*
251 (Fig. 3G) and *hoxd13* (Fig. 3H) were strongly expressed in whole region of a blastema.
252 MFCS1-GFP expression (Fig. 3R) and *gfp* transcript expression (Fig. 3F) are robustly
253 distributed in the entire blastema, while *shh* expression is restricted to the posterior edge
254 of the blastema (Fig. 3E). Therefore, the MFCS1-GFP expression domain is larger than
255 the *shh* expression domain, especially in a regenerating limb. Transcriptional repression
256 by a silencer or defined regulation of chromatin conformational change resulting in the
257 proximity of MFCS1 and the *shh* promoter (Amano et al., 2009) may further restrict
258 endogenous *shh* expression to the posterior edge of the blastema. Even though *shh*
259 expression is spatially narrower than the GFP expression pattern, the expression pattern
260 of MFCS1-GFP covers the *shh*-expressing domain and coincides with the expression
261 domains of upstream regulators represented by *hoxd13* and *dusp6*.
262

263 **MFCS1 is inactive in a limb blastema of a metamorphosed froglet despite *hoxd13***
264 **expression**

265 In contrast to a young tadpole, a limb of a metamorphosed froglet does not express *shh*
266 after amputation, and amputation results in regeneration of a cartilaginous spike, while
267 an amputated limb of a froglet forms a regeneration blastema (Endo et al., 2000; Suzuki
268 et al., 2006; Yakushiji et al., 2007). Hypermethylated status of genomic DNA at the
269 MFCS1 enhancer strongly suggested that this enhancer is inactivated in a froglet limb:
270 the enhancer inactivation implies transcriptional inactivation of *shh* in the limb (Yakushiji
271 et al., 2007). To examine whether inactivation of the enhancer occurs after
272 metamorphosis, we examined MFCS1-GFP expression in limb regeneration of a froglet.
273 From the early stage (early bud) blastema to the late stage rod-shaped blastema, no
274 specific GFP was observed in the blastema, while non-specific fluorescence was
275 sometimes observed in both MFCS1-GFP Tg froglets and wild-type ones (controls) (Fig.
276 3M-P and Fig. S3). Therefore, we conclude that MFCS1 is inactivated completely or is
277 much more inactive in the blastema of a froglet limb than in that of a young tadpole.
278 We then examined expression of upstream regulators of MFCS1. By 10 dpa, an

279 amputated limb of a froglet forms a cone-shaped blastema and the blastema already starts
280 expressing *fgf-8*, *fgf-10*, *msx1*, and *hand2,* genes that are involved in maintenance/
281 induction of *shh* expression in a limb bud (Suzuki et al., 2005; Yakushiji et al., 2007). In
282 addition, Yakushiji et al. (2007) reported hypermethylation status of genomic DNA in a
283 limb blastema at 10 dpa in a froglet. Therefore, we chose blastema at 10 dpa and examined
284 gene expression at that time point. Similar to the results of a study by Satoh et al. (2006)
285 for the blastema at a later time point (14 dpa), *hoxd13* was broadly expressed in the
286 blastema at 10 dpa (Fig. 3D). *dusp6* was also expressed in the blastema (Fig. 3C), while
287 no *shh* (and *gfp*) expression was detected in the blastema as Yakushiji et al. (2007)
288 reported previously (Fig. 3A, B). The expression of *hoxd13* and *dusp6* in a froglet
289 blastema suggests that upstream regulators of MFCS1 (5' Hoxd and ETS transcription
290 factors) as well as *hand2* (Yakushiji et al., 2007) are not absent in the froglet blastema.
291 Thus, MFCS1 in a froglet limb appears to be unable to respond to positive regulators
292 represented by Hoxd13.
293
294 **Higher DNA methylation level of MFCS1 may inactivate the enhancer's**
295 **responsiveness to Hoxd13 in a froglet limb**
296 To confirm the potential responsiveness of MFCS1 to the upstream regulators, we
297 conducted luciferase assays *in vitro* using MFCS1 with the *shh* promoter. The *luc2*
298 reporter under control of the MFCS1 enhancer with either *shh* or a minimum promoter
299 (Fig. 4A, upper diagram) was prepared. A local alignment of the highly conserved
300 sequences from different species revealed that there are multiple putative Hox-binding
301 motifs in the conserved sequences (Fig. 4A, see also Fig. S4). The full lengths of *Hoxd13*,
302 *Fgf2*, *Fgf4*, and *Fgf8* were flanked with the CMV promoter as gene expression constructs.
303 MFCS1-*shh* promoter-*luc2* or MFCS1-minP-*luc2* reporter constructs were co-transfected
304 with various combinations of the gene expression constructs into NIH3T3 cells. The
305 reporter activities of cells transfected with MFCS1-shh promoter-*luc2* were significantly
306 increased when co-transfecting with *Fgf2* (1.4-fold vs. mock), *Fgf4* (1.5-fold vs. mock),
307 *Fgf8* (1.3-fold vs. mock), or *Hoxd13* (2.3-fold vs. mock) (Fig. 4B). The combination of
308 *Fgf4* and *Hoxd13* additively affected MFCS1-driven gene activation (1.1-fold vs. *Hoxd13*
309 alone). Meanwhile, transfection of the MFCS1-*luc2* construct containing minP had did
310 not result in significant activation of the luciferase reporter even in the presence of any

311 expression constructs. When the MFCS1-*luc2* was artificially methylated by CpG
312 methyltransferase (M.SssI) in advance, the responsiveness of the reporter to Hoxd13 was
313 strongly suppressed by ~260 fold (Fig. S5). Taken together with the highly methylated
314 status of MFCS1 in a *Xenopus* froglet limb (Yakushiji et al., 2007), this result raises the
315 possibility that DNA methylation of MFCS1 inactivates its responsiveness to Hoxd13.
316 Genomic DNA methylation leads to chromatin condensation, inhibits access of
317 transcription factors to the enhancer sequence and finally results in transcriptional
318 repression (Bird and Wolffe, 1999). To further examine whether a high methylation level
319 of MFCS1 exerts a suppressive effect on gene expression, we investigated the
320 methylation status of MFCS1 as the transgene in MFCS1-GFP Tg tadpoles and froglets.
321 Within an MFCS1 highly conserved sequence in amphibians, there are nine CpG sites
322 both in *X. tropicalis* and *X. laevis* (Fig. S6). Firstly, we examined DNA methylation status
323 of MFCS1 in wild-type tadpoles and froglets by bisulfite sequencing as reported by
324 Yakushiji et al. (2007) and obtained consistent results: While limb buds of young tadpoles
325 have a lower methylation level (26.3%), froglet limbs have a higher methylation level
326 (87.2%) as shown in Fig. 4C. We then examined the methylation level of the MFCS1
327 sequence as the transgene in MFCS1-GFP Tg (line 1). Again, froglet limbs have a higher
328 methylation level (17.2%) than that in limb buds of young tadpoles (2.6%) (Fig. 4C).
329 After limb amputation in a froglet and after completion of spike regeneration, a higher
330 methylation level than that in tadpoles was still found in the Tg regenerates (line 1,
331 14.4%) as well as in wild-type ones (Fig. S7). The nine CpG sites in MFCS1 (highly
332 conserved sequence in amphibians) as the transgene were susceptible to methylation to
333 some extent also in line 2 (Fig. S7). Although the basal methylation level differs between
334 endogenous MFCS1 and transgene MFCS1, the increase in the methylation level of the
335 transgene MFCS1 after metamorphosis also correlated with the ontogenetic inactivation
336 of gene expression under control of the enhancer.
337

338 **Conclusions**

339 In this study, we visualized MFCS1 activity in limb regeneration of an amphibian.
340 MFCS1-GFP Tg *Xenopus* revealed that MFCS1 enhancer is re-activated specifically in
341 the regenerating blastema for a patterned limb in a young tadpole but not in the blastema
342 for a hypomorphic limb in a froglet. MFCS1 seems to be activated by Hoxd13 in a tadpole

343 but seems to be unable to respond to Hoxd13 in a froglet. An ontogenetic increase in the
344 MFCS1 methylation level may block the function of Hoxd13 (and other positive
345 regulators) as a transcriptional activator(s) to the enhancer so that transcription of a gene
346 under control of the enhancer is inactivated (Fig. 4D). For improvement of regenerative
347 capacity in a froglet limb by re-activation of *shh* expression, removal of epigenetic
348 silencing of MFCS1 would be important. The combinational application of an inhibitor
349 of DNA methyltransferase and a histone deacetylase inhibitor stimulated *shh* expression
350 in cultured blastema cells of a froglet limb in a previous *in vitro* study (Yakushiji et al.,
351 2009), suggesting that application of such reagents affecting epigenetic modification
352 could be hopeful if the treatment condition is optimized *in vivo*. As an alternative
353 approach, CRISPR-based epigenome editing that removes DNA methylation (e.g., Nuñez
354 et al., 2021) may be effective. In these approaches, the MFCS1-GFP Tg line would be
355 useful for evaluating the effectiveness. The enhancer activation is easily detected by GFP
356 and it would thus be possible to screen chemicals that remove epigenetic silencing of the
357 enhancer in a large scale. If regeneration of a well-patterned limb is induced in a froglet
358 by re-activation of MFCS1, it would be a precious model case for recovery of organ-level
359 regeneration in non-regenerative higher vertebrates by manipulating epigenetics.
360

361 # MATERIALS AND METHODS

362 **Transgene construction and *in situ* hybridization**

363 A 3,164-bp MFCS1 enhancer sequence was amplified from *Xenopus tropicalis* genomic
364 DNA prepared from a Nigerian line with primers (XtMFCS1-Fw and Xt-MFCS1-Rv;
365 Table S3). The PCR product was cloned into the pCRII-TOPO vector (Invitrogen) and
366 sequenced. The *Xenopus laevis shh* promoter (Yakushiji et al., 2007) was cloned into a
367 reporter construct that contains an EGFP expression cassette. Then the MFCS1-*shh*
368 promoter-*egfp* was generated by inserting the 3,164-bp MFCS1 enhancer sequence
369 upstream of the *shh* promoter (Fig. 1A). For synthesis of probes for *in situ* hybridization,
370 a *hoxd13* cDNA clone, BC080434, inserted in pCMV-SPORT6 was obtained from
371 Dharmacon. The *hoxd13*/ pCMV-SPORT6 plasmid was linearized with SalI and
372 transcribed with T7 RNA polymerase. DIG-labeled RNA probes of *hoxd13*, *shh* (Endo et
373 al., 1997), *gfp* (Hayashi et al., 2015) and *dusp6* (Gomez et al., 2005) were prepared

374 according to the protocol of the manufacturer (Roche). To prepare serial cryosections, the
375 specimens were fixed in MEMFA (0.1 M MOPS at pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$,
376 3.7% formaldehyde) overnight at room temperature, embedded in OCT compound
377 (Sakura), and serially sectioned at 8-10 μm by a cryostat. *In situ* hybridization on frozen
378 sections was performed according to Yoshida et al. (1996) with slight modifications.
379 Sections were observed under an upright microscope (Olympus BX53) with an objective
380 lens (Olympus UPlan PLN 10X) and were photographed by a digital camera (Leica
381 DFC7000T).
382
383 **Animal husbandry and transgenesis in *Xenopus laevis***
384 Wild-type *Xenopus laevis* adult frogs were purchased from domestic animal venders,
385 Watanabe Zoushoku (http://www5d.biglobe.ne.jp/~zoushoku/top.htm) and Hamamatsu
386 Seibutsu Kyozai (http://www.h-seibutsu.co.jp/index.html). Founder (G0) MFCS1-GFP
387 Tg *Xenopus laevis* embryos were generated by a REMI technique (Kroll and Amaya,
388 1996) using oocyte extract instead of egg extract (Hirsch et al., 2002; reviewed in Ogino
389 and Ochi, 2009). MFCS1-GFP N1 Tg *Xenopus laevis* lines were obtained by crossing
390 sexually mature MFCS1-GFP G0 frogs with wild-type frogs. Tadpoles and froglets were
391 reared at 22–23°C in dechlorinated tap water. The rearing containers were cleaned daily,
392 and the tadpoles were fed powdered barley grass (Odani Kokufun, Japan or Yamamoto
393 Kanpoh, Japan). At stage 58 (Nieuwkoop and Faber, 1994), the feeding was ceased until
394 metamorphosis was completed. After metamorphosis, the froglets were fed dried tubifex
395 every other day. All surgery was performed under ethyl-3-aminobenzoate anesthesia, and
396 all efforts were made to minimize suffering. When tadpoles and froglets needed to be
397 euthanized, they were immersed in 0.05% ethyl-3-aminobenzoate (Tokyo Chemical
398 Industry, 886-86-2). When adult male frogs were sacrificed to excise their testes for
399 transgenesis, 2% ethyl-3-aminobenzoate dissolved in pure water was injected into the
400 intracardiac cavity (400 μg of ethyl-3-aminobenzoate/1 g of animal). After the male frog
401 was confirmed to be completely unconscious, its testes were excised with a blade, and
402 then the unconscious frog was put in a freezer for euthanization. All animal care and
403 experimentation procedures were conducted in accordance with the animal care and use
404 committee guidelines of Tohoku University (2015LsA-023) and Hirosaki University
405 (A15003, A15003-1).

406

407 **Observation of developing and regenerating limbs, limb amputation and 180°**
408 **rotation of a limb bud**

409 For observation of limb development and limb regeneration in tadpoles, we used hindlimb
410 buds since it is much easier to observe and to operate on hindlimb buds than forelimb
411 buds due to their size and accessibility. To observe the regenerating limb bud (blastema)
412 of a tadpole, tadpoles at stage 54 were anesthetized with 0.025% ethyl-3-aminobenzoate
413 and hindlimb buds were amputated using ophthalmologic scissors at the presumptive
414 ankle level (according to the outside view and a fate map by Tschumi, 1957). For
415 preparation of a 180° rotated limb bud, Nile Blue A particles were embedded into the
416 anterior side of the distal tip of a stage 53 hindlimb bud with a tungsten needle as the
417 reference point indicating the original AP axis of the graft according to Cameron and
418 Fallon (1977). Subsequently, the limb bud was amputated at the presumptive ankle level
419 (Tschumi, 1957) and then the distal limb bud tip was rotated 180° (not rotated for control
420 limbs) on the proximo-distal axis, returned to the stump, and fixed with tungsten pins
421 according to Cameron and Fallon (1977) and Endo et al. (1997). After metamorphosis,
422 the resulting limbs were fixed in 10% formalin in Holtfreter's solution and stained with
423 0.1% Alcian blue as previously reported (Yokoyama et al., 1998). Then digit identity was
424 determined by the number of phalanges in association with the presence/absence of a
425 claw (Cameron and Fallon, 1977). To observe the blastema of a froglet, froglets were
426 anesthetized with 0.05% ethyl-3-aminobenzoate and then forelimbs were amputated
427 through the distal zeugopodium with ophthalmologic scissors and the amputation surface
428 was trimmed to be flat. For observation of limb regeneration in froglets, we used
429 forelimbs since hindlimb amputation often results in drowning of the froglets.

430

431 **Imaging of whole-mount limb samples**

432 Tadpoles and froglets anesthetized with 0.025% or 0.05% ethyl-3-aminobenzoate were
433 observed under a fluorescence stereomicroscope (Nikon SMZ18) with a 0.5x objective
434 lens using a GFP longpass filter (if in a fluorescent view) and photographed by a digital
435 camera (Olympus DP74 or DP73). For confocal microscopy (Fig. 1D, E and Fig. S1B,
436 C), anesthetized tadpoles were each placed on a 35-mm glass-bottom dish (Iwaki, Japan)
437 so that the dorsal surface of the hindlimb bud was tightly attached to the glass-bottom.

438 The prepared samples were observed under an FV3000 confocal laser scanning
439 microscope (Olympus) equipped with an Olympus UPlanSApo 20× dry objective lens.
440 Fluorescence between 500 nm and 600 nm was detected by a GaAsP photo multiplier
441 tube.
442
443 **Genome sequence analysis**
444 The 449-b genomic sequences corresponding to a conserved core region of human
445 MFCS1 (hg38, chr7: 156,791,242－156,791,690) and its orthologous sequences in the
446 mouse (mm39, chr5: 29,520,033–29,520,474), chicken (galGal6, chr2: 8,553,351–
447 8,553,801), *X. tropicalis* (xenTro10, chr6: 17,346,249–17,346,700) and *X. laevis* (J-strain
448 10.1, Chr6L: 15,900,483–15,900,931) were downloaded from the UCSC Genome
449 Browser and Xenbase (Karolchik et al., 2003; Fortriede et al., 2020). These sequences
450 were aligned using MUSCLE (Edgar, 2004). Searches for potential transcription factor-
451 binding sites were performed with transcription factor-binding motifs collected from the
452 TRANSFAC and JASPAR databases (Wingender, 2008; Castro-Mondragon et al., 2022).
453 The *X. tropicalis* MFCS1 element used for our functional analyses contains a 12-
454 thymidine repeat in place of the 14-thymidine repeat underlined in the alignment (Fig.
455 4A, underlined). This difference may be because of a polymorphism between the strain
456 used for our study and that used for the genome sequencing project (Igawa et al., 2015).
457
458 **Reporter construction, cell culture and luciferase assay**
459 The highly conserved 998-bp core sequence of *Xenopus tropicalis* MFCS1 was amplified
460 by PCR with specific primers (Table S3) and inserted into the Acc65I and XhoI sites of
461 the pGL4.23 vector (Promega, E8411). Then the minimal promoter of the pGL4.23 vector
462 was replaced with a 486-bp sequence of *Xenopus laevis shh* promoter (Fig. 4A). It should
463 be noted that the 14-thymidine repeat in *X. tropicalis* MFCS1 sequence from the UCSC
464 Genome Browser (Fig. 4A, underlined) was shortened to a 12-thymidine repeat in the
465 transgene construct (Fig. S6) and the reporter construct for the luc assay. pGL4.74
466 containing the *Renilla* luciferase gene was used for normalization of reporter expression
467 (Promega, E6921). Coding sequences of mouse *Hoxd13*, *Fgf2*, *Fgf4* and *Fgf8* were cloned
468 into the pcDNA3.1 vector (ThermoFisher, V79020). NIH3T3 cell line (RCB2767) was

469 obtained from the RIKEN BioResource Research Center. NIH3T3 cells were maintained
470 in Dulbecco's modified Eagle's medium (ThermoFisher, 11960-044) containing 10%
471 fetal bovine serum, 1x GlutaMax and 100 μg/ml penicillin–streptomycin. Cells at 70–
472 90% confluency were transfected with equal amounts of luciferase reporter and gene
473 expression plasmids using Lipofectamine 3000 (ThermoFisher). Luciferase activity in the
474 cell lysates was measured using a Dual-Luciferase Reporter Assay System (Promega)
475 according to the manufacturer's protocol. For statistical analysis, Student's *t*-tests (two-
476 tailed) were applied when equal variance tests were passed.
477
478 **Bisulfite genomic sequencing**
479 Presumptive autopods of hindlimb buds (Tschumi, 1957) from thirty-five wild-type and
480 sixteen MFCS1-GFP Tg (line 1) tadpoles at stage 54 were collected. Forelimb autopods
481 from five wild-type and six MFCS1-GFP Tg (line 1) froglets were collected. Spike
482 regenerates from two MFCS1-GFP Tg (line 1) and two same Tg (line 2) frogs were
483 collected. Then genomic DNA was extracted from these tissue samples. The genomic
484 DNA was treated with sodium bisulfite using an EZ DNA Methylation-Gold Kit
485 following the protocol of the manufacturer (Zymo Research). DNA was amplified by 30
486 cycles of a three-step PCR (98°C for 10 sec, 60°C for 30 sec and 68°C for 30 sec) with
487 MFCS1_BS_Fw and MFCS1_BS_Rv primers (Table S3) using KOD -Multi & Epi-
488 (Toyobo). Each product was cloned into the pANT vector (Nippon Gene) or pGEM-T
489 easy (Promega) and sequenced with M13F-pUC or M13R-pUC primers (Table S3).
490

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization: K.T., H.Y.; Methodology: T.A., N.Y.-K., J.S., Y.K., H.Y.; Validation: T.A., N.Y.-K., H.Y.; Formal analysis: T.A., H.Ogino, H.Y.; Investigation: R.T., T.H., T.A., S.I., C.Y., S.N., K.I., A.K., H.Ochi, H.Ogino, N.Y.-K., Y.K., H.Y.; Resource: T.A., A.K., H.Ochi, H.Ogino, N.Y.-K., Y.K., K.T., H.Y.; Writing – original draft preparation: T.A., H.Ogino, J.S., Y.K., H.Y.; Writing – review and editing: A.K., H.Ochi, H.Ogino, N.Y.-K., K.T., H.Y.; Visualization: R.T., T.H., T.A., S.I., H.Ogino, N.Y.-K., H.Y.; Supervision: K.T., H.Y.; Project administration: H.Ogino, K.T., H.Y.; Funding acquisition: K.T., H.Y.

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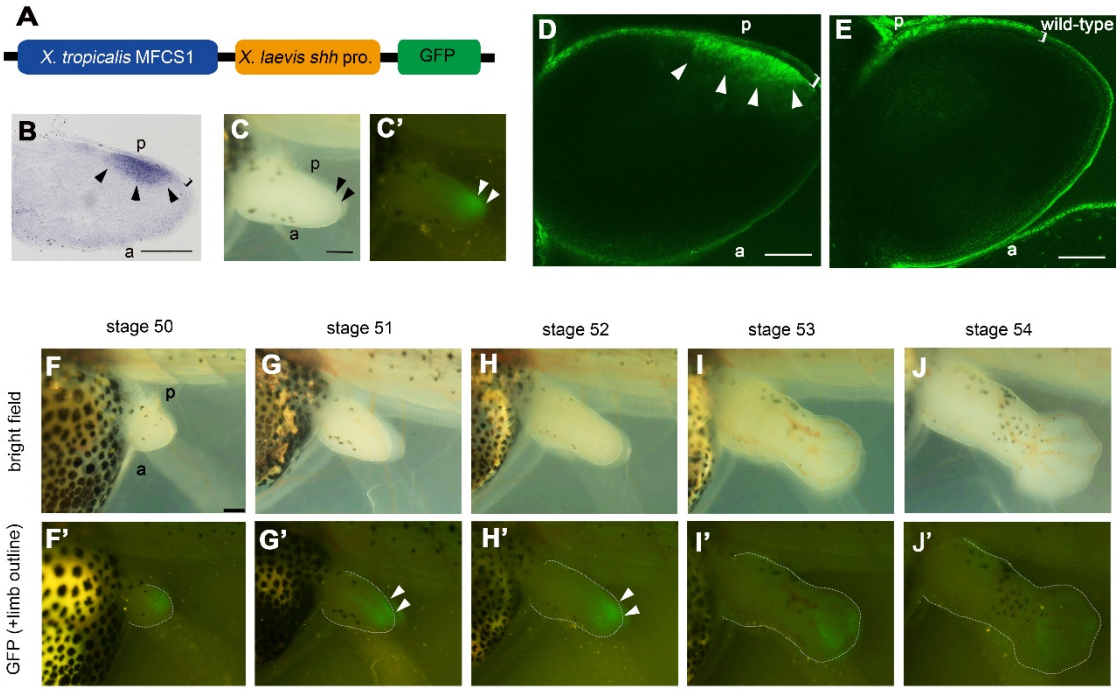
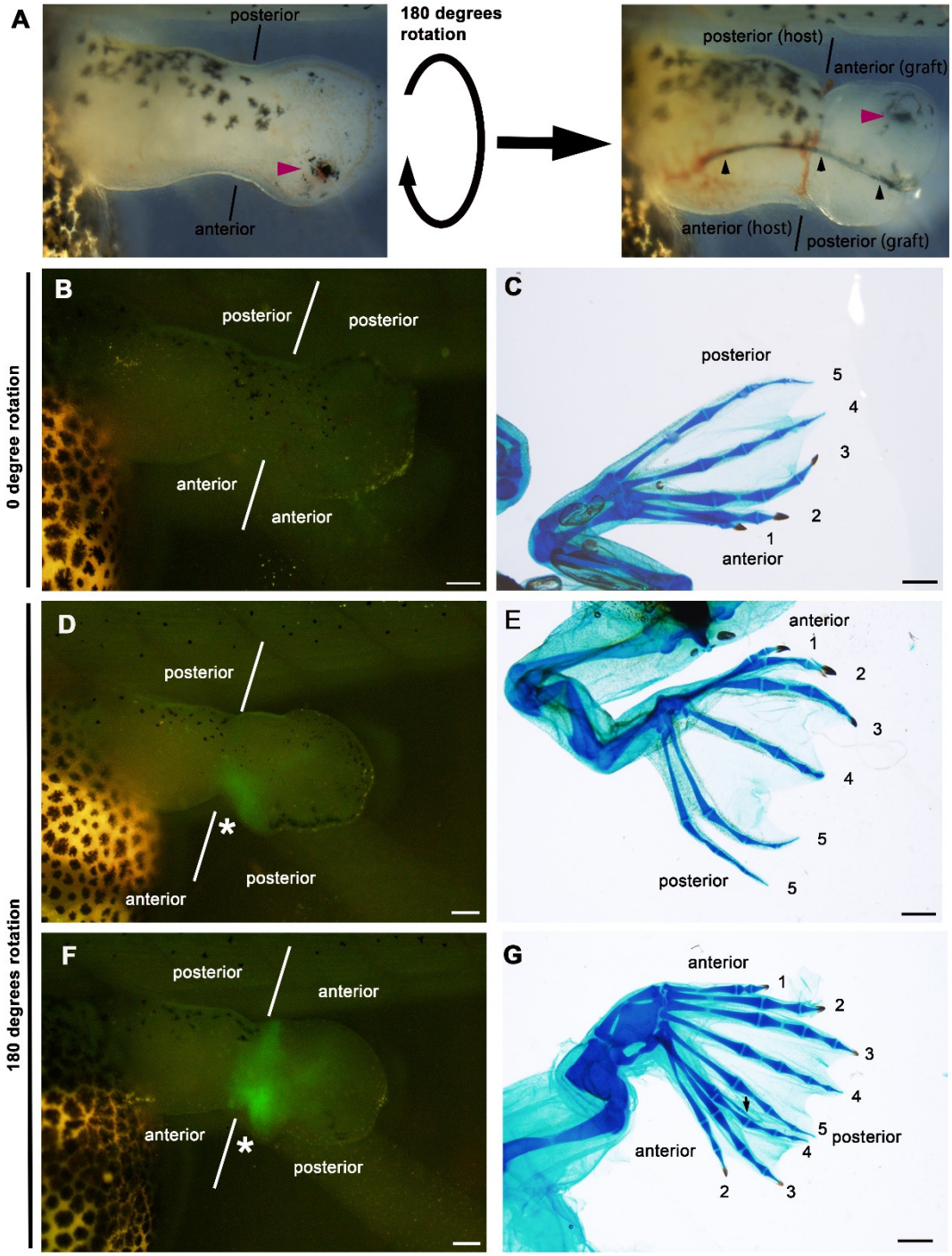



**Fig. 1. MFCS1-GFP expression coincides with *shh* expression in a developing limb bud.** (A) Map of the MFCS1-GFP transgene. (B) *shh* expression in a limb bud at stage 52. (C, C') A limb bud of an MFCS1-GFP tadpole at stage 51. Bright-field (C) and fluorescent (C') images. (D, E) Optical-section images by confocal microscopy (approximately 60-70 μm in depth from the dorsal surface) of the right limb bud of an MFCS1-GFP tadpole (D) and that of a wild-type tadpole (E) at stage 51. Bright-field (F-J) and fluorescent (F'-J') images of the left limb bud of the same MFCS1-GFP tadpole. Limb buds are outlined by dotted lines in the fluorescent images. All of the MFCS1-GFP tadpoles shown are line 1. a, anterior: p, posterior. Arrowheads show *shh* expression (B) or GFP expression (D, E, G', H') at the posterior side. Brackets indicate the epidermal cell layer. Bars = 100 μm for (B, D ,E) and 200 μm for (C, C', F-J, F'-J').




683

684 **Fig. 2. MFCS1-GFP expression precedes limb duplication along the AP axis in 180°**
685 **rotated limb buds.** (A) 180° rotation of a limb bud at stage 53. A magenta arrowhead
686 indicates an embedded Nile blue A particle. Note the inverted position of the particle
687 before (lower side) and after (upper side) grafting. Black arrowheads indicate a tungsten
688 pin. (B, C) A 0° rotated limb bud at 6 days after grafting (B) and the resultant normal limb

689 formation (C). (D-G) 180° rotated limb buds. Rotated limb buds at 6 days after grafting
690 (D,F) resulted in supernumerary limb formation (E,G). Note that the sample with brighter
691 GFP (F) resulted in more supernumerary limbs (G). All of the operated MFCS1-GFP
692 tadpoles are line 1. A pair of lines indicates the host-graft boundary. An asterisk indicates
693 ectopic GFP. An Arabic number indicates the digit identify. A black arrow shows an
694 unidentifiable digit. Bars = 200 μm for (B, D, F) and 1 mm for (C, E, F).
695

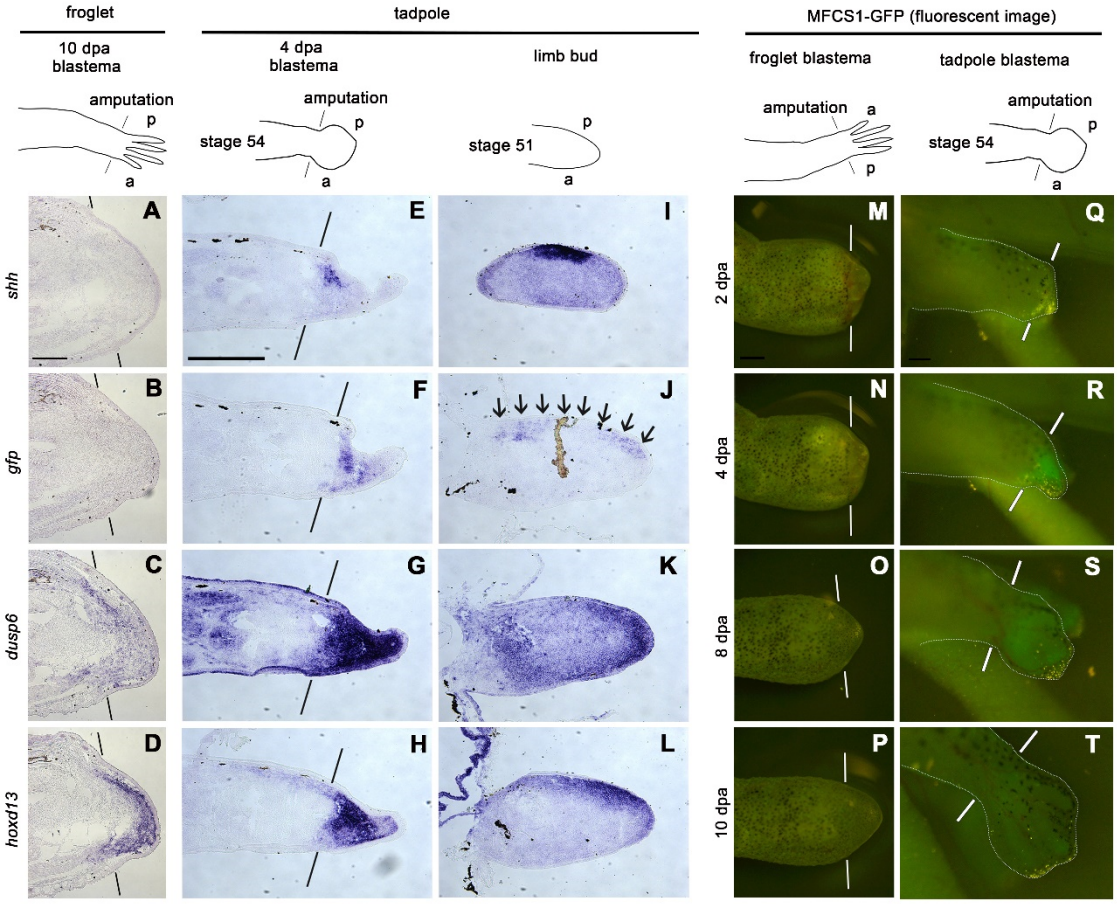



**Fig. 3. Comparison of gene expression patterns and MFCS1-GFP expression pattern in a regenerating limb in a young tadpole and a froglet.** (A-D) Adjacent sections of a blastema of a froglet. n=5 of 5 blastemas from a total of five froglets of line 1. (E-H) Adjacent sections of a blastema of a tadpole. (I-L) Adjacent sections of a limb bud. To guarantee an accurate comparison of gene expression levels, limb bud and blastema sections were treated in exactly the same way. n=9 of 9 blastemas from a total of five tadpoles of line 1 and 6 of 6 blastemas from a total of three tadpoles of line 2, respectively. n=10 of 10 limb buds from a total of five tadpoles of line 1 and 6 of 6 from a total of three tadpoles of line 2, respectively. (M-P) A blastema of a froglet of line 1. See also Fig. S3 for details. (Q-T) A blastema of a tadpole of line 1. The blastema and the stump are outlined by a dotted line. See also Fig. S2 for details. a, anterior: p, posterior. A pair of lines indicates the amputation level. Arrows indicate *gfp* expression at the posterior side. All pictures shown in Fig. 3 are samples of line 1. Bar = 200 μm for (A-L, Q-T) and 500 μm for (M-P).

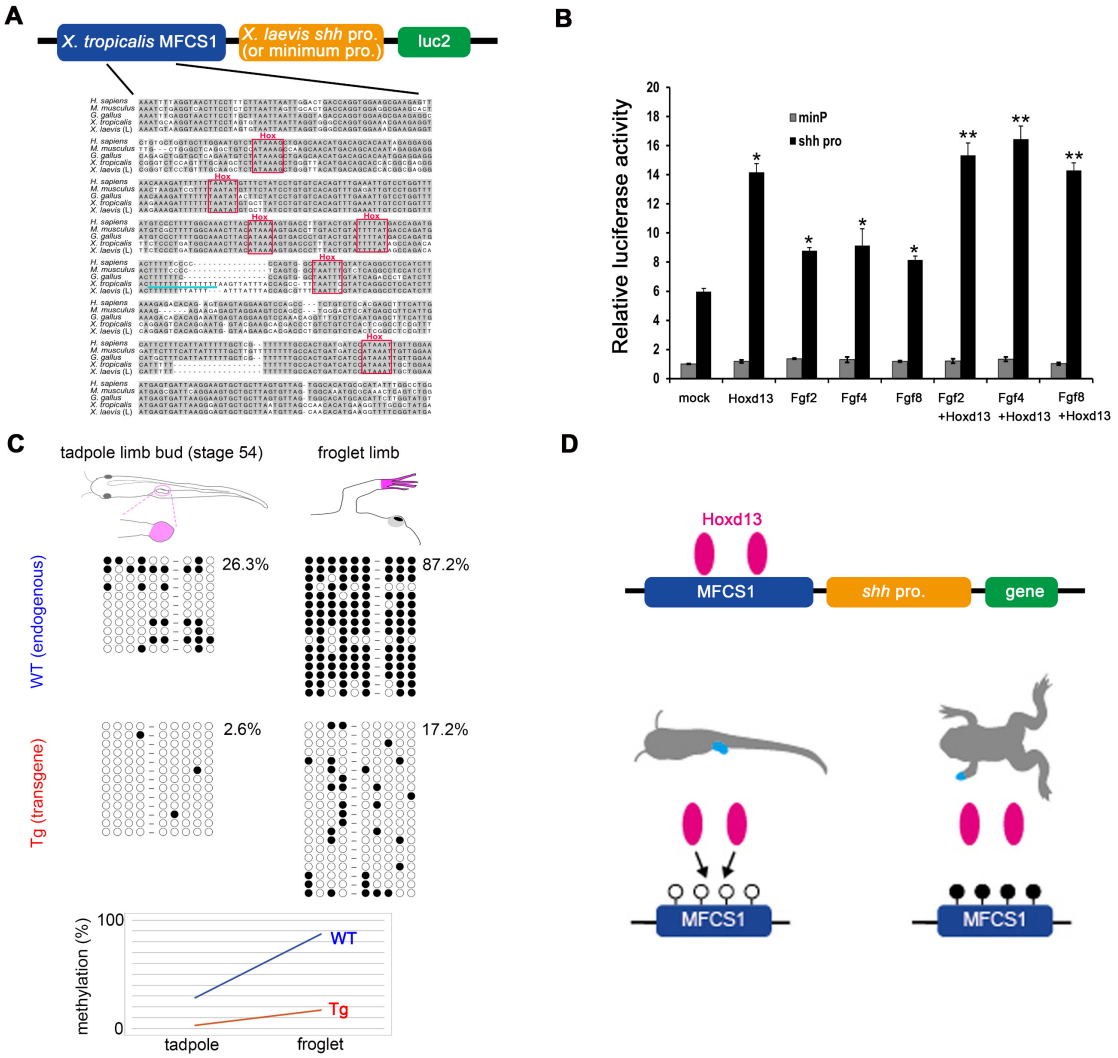
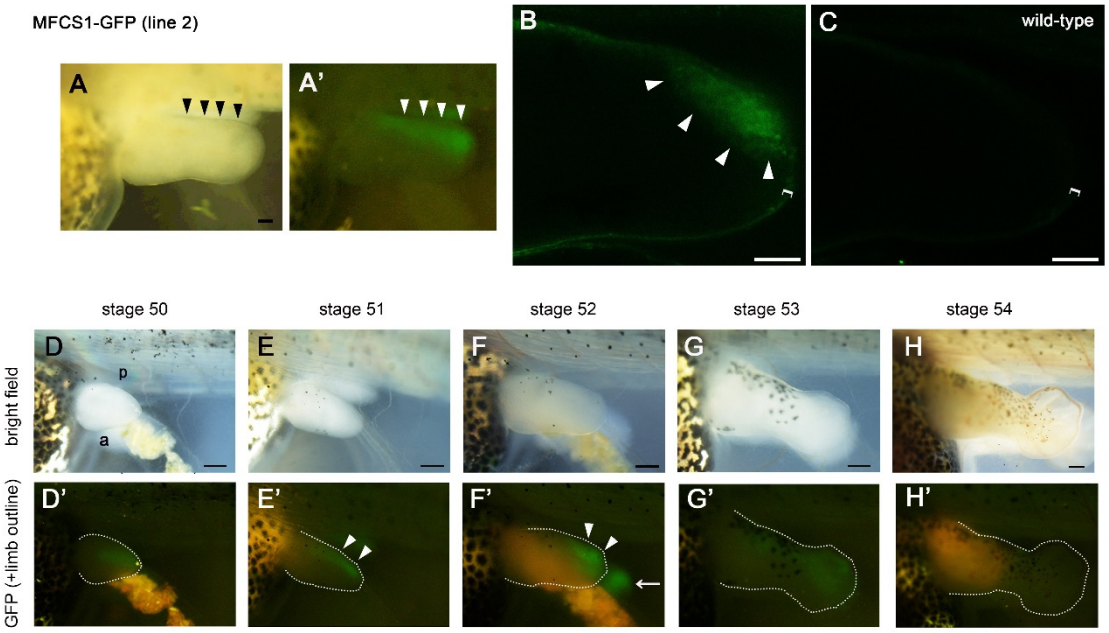



**Fig. 4. Transactivation and DNA methylation of *Xenopus* MFCS1 enhancer.** (A) Upper panel: Map of MFCS1-luc2 used for assays in (B). Lower panel: Alignment of MFCS1 highly conserved sequences. Nucleotides identical in more than two sequences are shaded in grey. Conserved Hox-binding motifs are boxed. A light bule line underlies the 14-thymidine repeat in *X. tropicalis* (See Materials and Methods for details.). (B) Firefly luciferase activity of MFCS1-luc2 using a minimum promoter (minP) or *shh* promoter (shh pro) was normalized to *Renilla* luciferase control activity. The mean value of mock samples by the control construct with the minimum promoter was adjusted to 1.0. Error bars: standard deviation from the mean (n=3). *Significant increase from mock (p<0.01). **Significant increase in Fgf2+Hoxd13, Fgf4+Hoxd13 and Fgf8+Hoxd13 from sole Fgf2, Fgf4 and Fgf8, respectively (p<0.01). (C) Methylation pattern of endogenous and transgene MFCS1 (line 1). Samples were obtained from the autopod (pink). Each

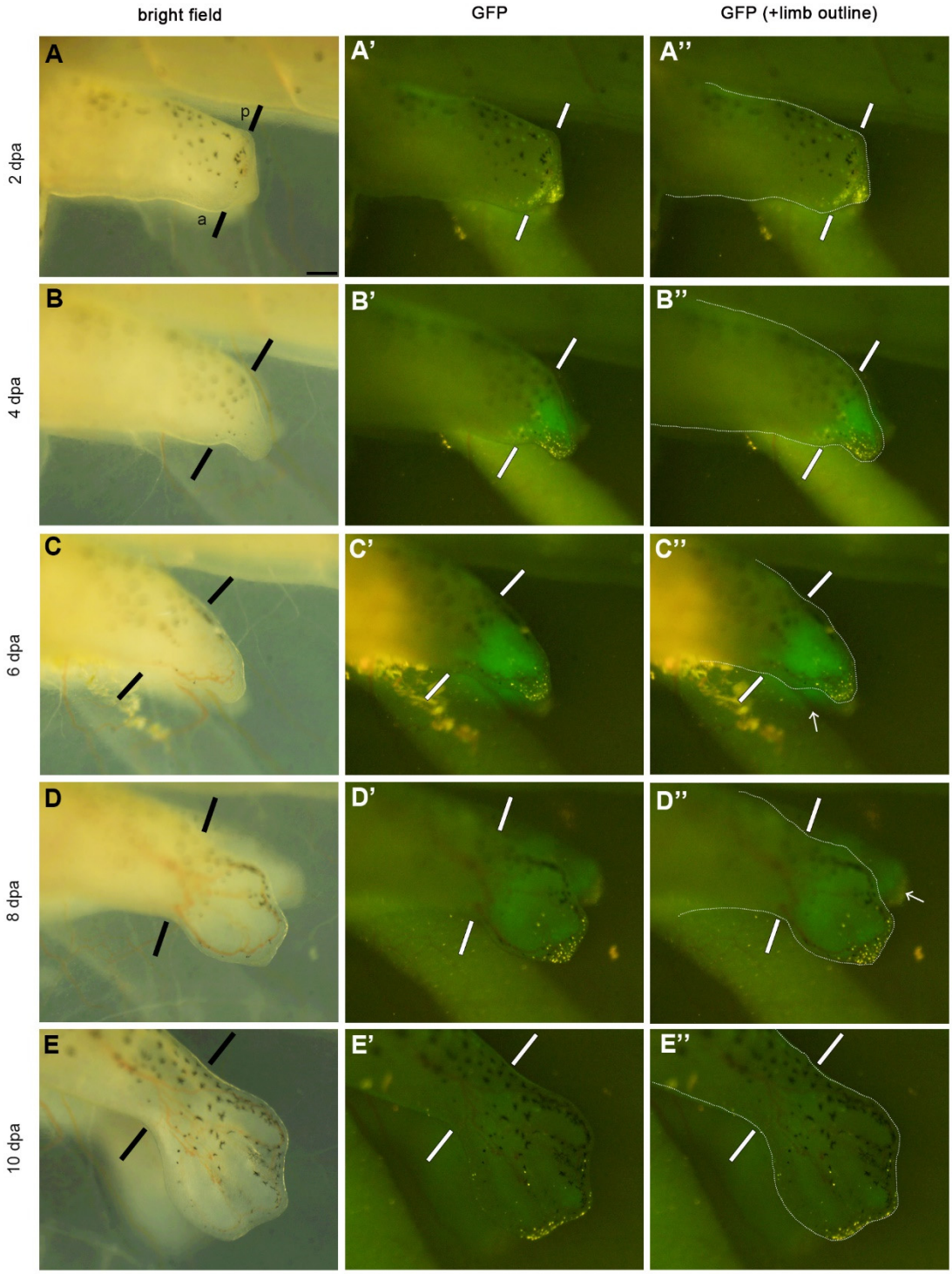
725 circle corresponds to a CpG site (see Fig. S6). Lower panel: Increases in average
726 methylation rate of the MFCS1 sequences after metamorphosis. (D) Model for epigenetic
727 silencing of gene expression through DNA methylation of MFCS1. Hoxd13 activates
728 hypomethylated MFCS1 and results in transcription of a gene under control of MFCS1
729 in the tadpole limb (left). However, Hoxd13 is unable to activate highly methylated
730 MFCS1 in a froglet limb and results in inactive transcription of the gene (right).
731




732

733 **Fig. S1. MFCS1-GFP expression pattern was recapitulated in line 2.** (A) A developing
734 limb bud of an MFCS1-GFP Tg tadpole of line 2 at stage 52. Bright-field (A) and
735 fluorescent (A') images of the same limb bud. (B, C) Optical-section images by confocal
736 microscopy (approximately 80 μm in depth from the dorsal surface) of the right limb bud
737 of an MFCS1-GFP Tg tadpole of line 2 (B) and a wild-type tadpole (C) at stage 52. GFP
738 expression was detected in the posterior side mesenchyme specifically in the MFCS1-
739 GFP Tg tadpole. Bright field (D-H) and fluorescent (D'-H') images of the limb bud of
740 the same Tg tadpole of line 2. Limb buds are outlined by dotted lines. GFP expression
741 was detected in the posterior side of limb buds at stage 51-52 and then gradually
742 diminished. a, anterior: p, posterior. Arrowheads show GFP expression (A, A', B, E', F')
743 at the posterior side of the limb buds. Brackets indicate the epidermal cell layer. A pair of
744 lines indicates the amputation level. Bars = 100 μm for (A, B, C), 200 μm for (D-H, D'-
745 H').

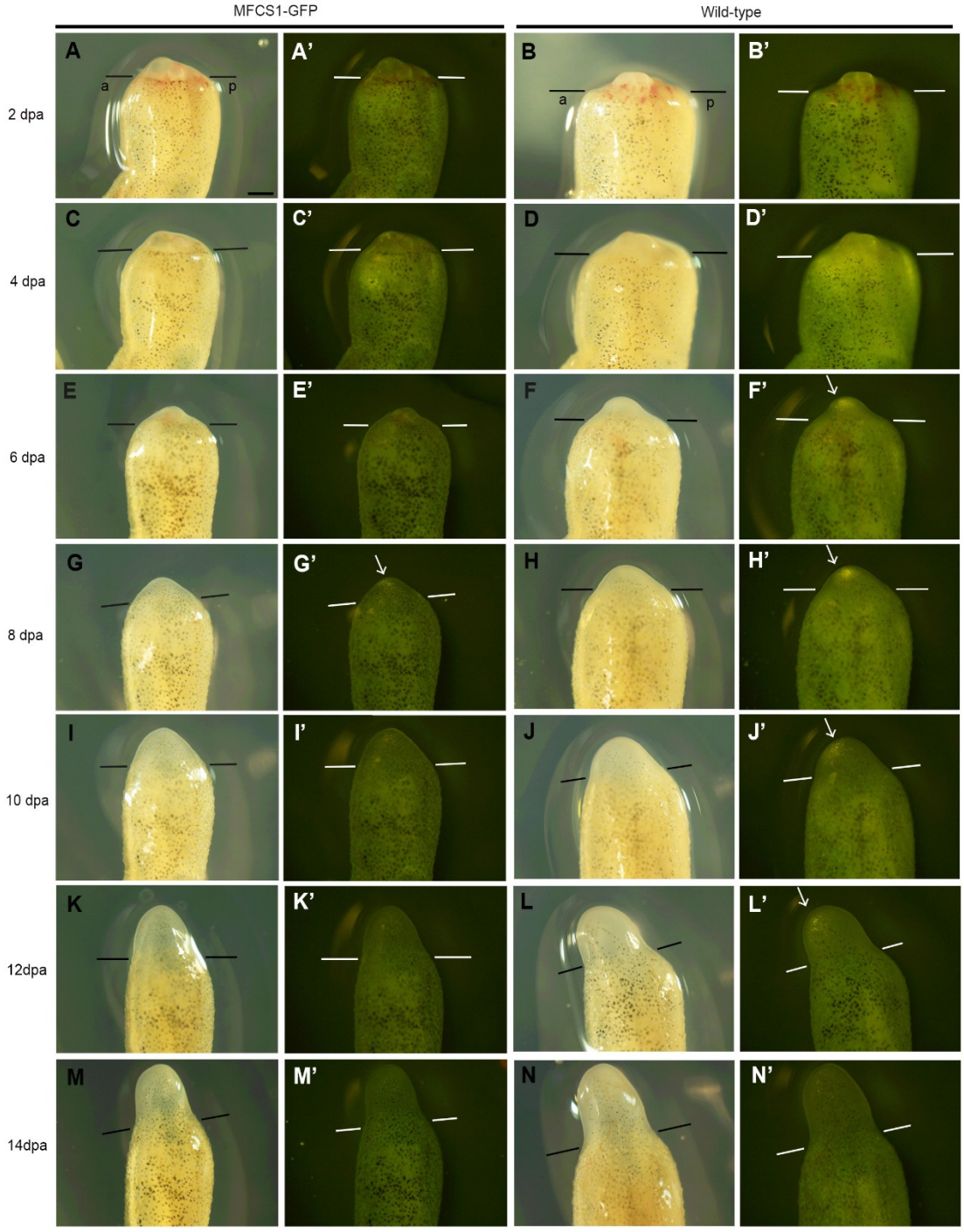
746




747

748 **Fig. S2. Spatio-temporal expression pattern of MFCS1-GFP in a regenerating limb**
749 **of a young tadpole.** All of the pictures are focused on a limb blastema of the same tadpole
750 (identical to Fig. 3Q-T) of line 1. Left and right limb buds were amputated at the
751 presumptive ankle level of a stage 54 tadpole. GFP fluorescence was distributed

752 throughout the regenerating limb blastema at 4 dpa and 6 dpa. (A'', B'', C'', D'', E''')
753 Limb blastemas and stumps are outlined by dotted lines. n=16 of 16 blastemas from a
754 total of ten MFCS1-GFP tadpoles of line 1 and 6 of 6 blastemas from a total of three
755 MFCS1-GFP tadpoles of line 2, respectively. A pair of lines indicates the amputation
756 level. Arrows show GFP fluorescence of the contralateral right limb blastemas. a,
757 anterior: p, posterior. Bar = 200 μm.
758




759

760 **Fig. S3. MFCS1-GFP expression is inactive in a limb blastema of a froglet.** A blastema
761 of the same froglet of line 1 (right two columns; identical to Fig. 3M-N) and a blastema
762 of the same wild-type froglet (left two columns) from 2 dpa to 14 dpa. No specific GFP
763 fluorescence was observed in the blastema of the Tg froglet at any timepoint. n=4 of 4
764 blastemas from a total of four froglets of line 1, 6 of 6 blastemas from a total of four

765 froglets of line 2 and 8 of 8 blastemas from a total of six wild-type froglets as controls,
766 respectively. A pair of lines indicates the amputation level. An arrow indicates non-
767 specific fluorescence commonly observed both in the Tg froglet and wild-type one. a,
768 anterior: p, posterior. Bar = 500 μm.
769

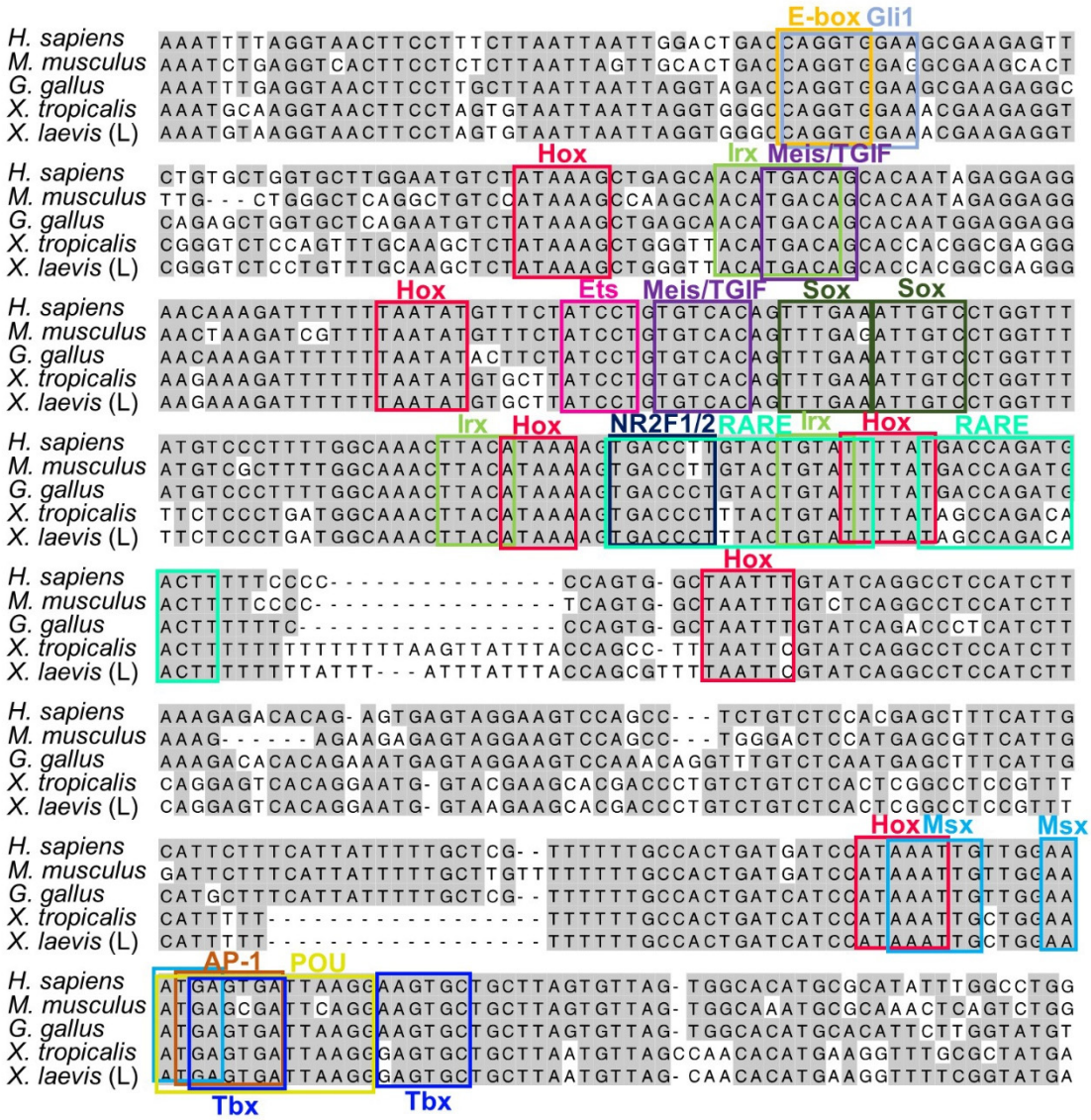



**Fig. S4. Alignment of MFCS1 highly conserved sequences (identical sequences shown in Fig. 4A) from the human, mouse, chick, *X. tropicalis* and *X. laevis*.** Nucleotides identical in more than two sequences are shaded in grey. Conserved transcription factor-binding motifs (Hox and other transcription factors) are boxed.

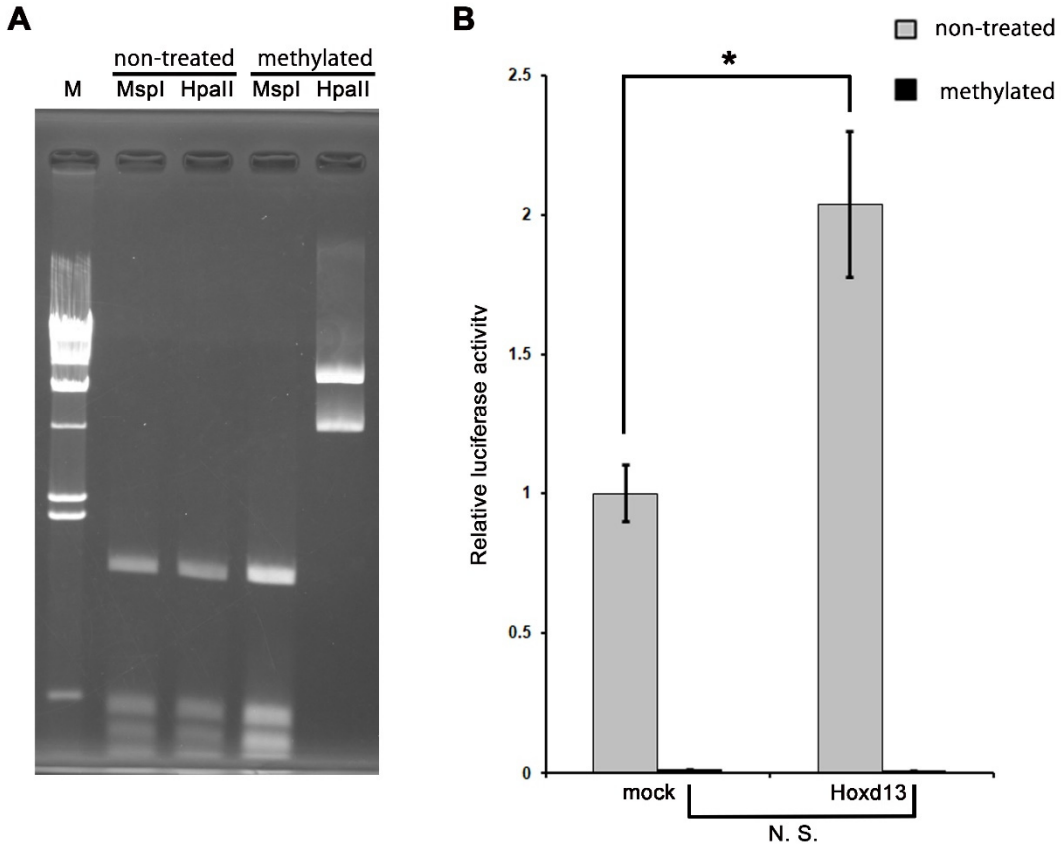



**Fig. S5. DNA methylation suppresses the response of MFCS1 to Hoxd13 *in vitro*.** (A) Methylation-sensitive restriction digestion visualized by agarose gel electrophoresis. Plasmid DNA of the MFCS1-*shh* promoter-*luc2* reporter was treated with CpG methyltransferase (M. SssI, NEB) according to the manufacturer's protocol. Methylation efficiency of the plasmid DNA was evaluated by digestion with methylation-sensitive (HpaII) and -insensitive (MspI) restriction enzymes. The CpG methylation prohibited the digestion of the plasmid DNA specifically with HpaII. M, l/HindIII marker. (B) Luciferase activity in NIH3T3 cells transfected with the methylated or non-treated MFCS1-luc2 reporter constructs and the *Hoxd13* expression construct. The mean value of luciferase activity in cells transfected with the non-treated reporter and mock expression constructs was set to 1.0. Error bars: standard deviation from the mean (n=3). While a significant increase of *Hoxd13*-induced luciferase activity was observed in cells transfected with the unmethylated reporter construct (*$p<0.01$), being consistent with Fig. 4B, there was no significant increase in cells transfected with the methylated reporter construct despite *Hoxd13* expression. N. S., not significant.

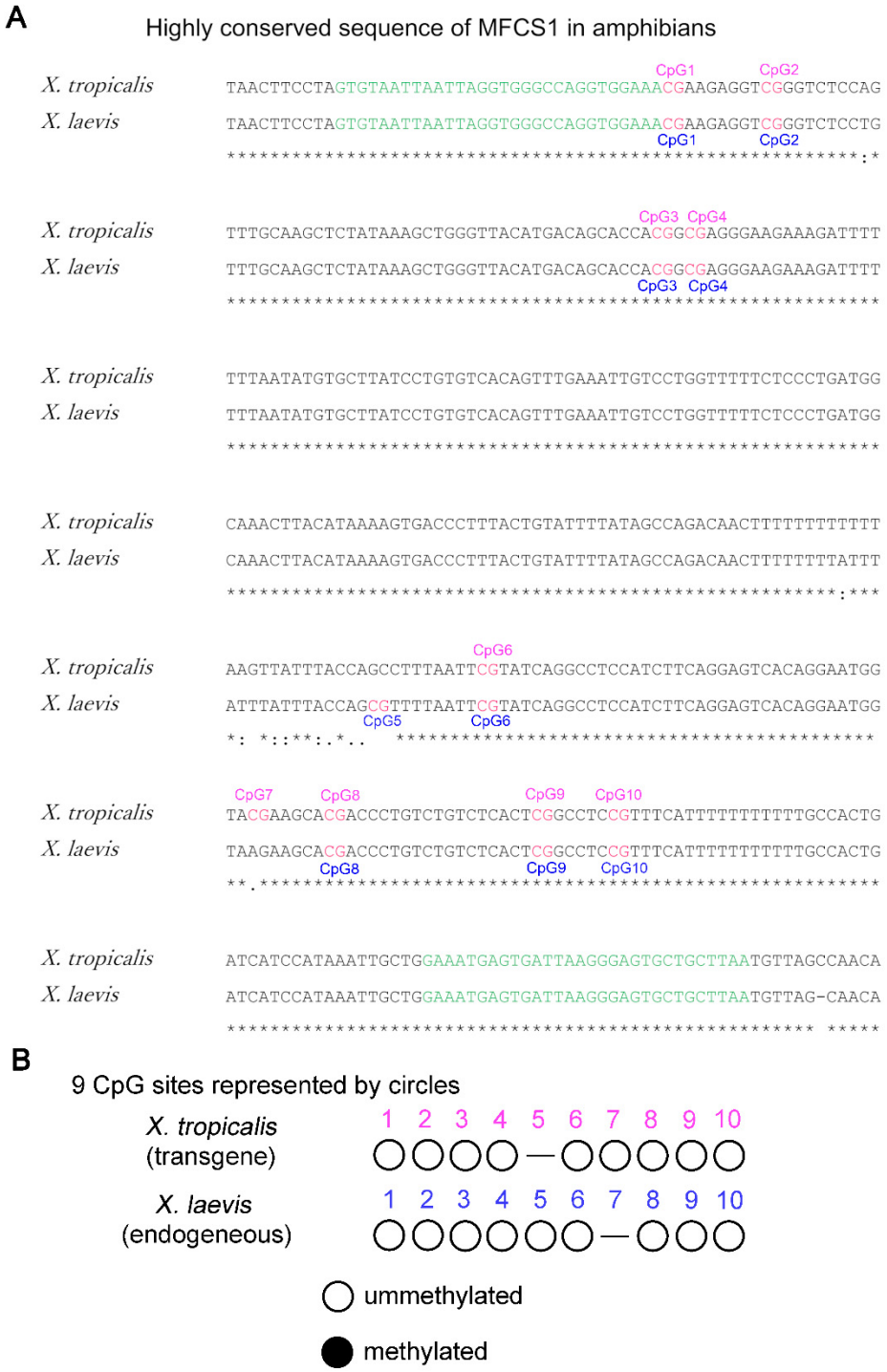



**Fig. S6. *X. tropicalis* and *X. laevis* have 9 CpG sites in the highly conserved sequence of MFCS1.** Alignment of MFCS1 highly conserved sequences between *X. tropicalis* and *X. laevis*. Green letters correspond to MFCS1_BS_Fw and MFCS1_BS_Rv primers for bisulfite sequencing (see also Table S3). Open and black circles represent unmethylated and methylated CpG sites, respectively.

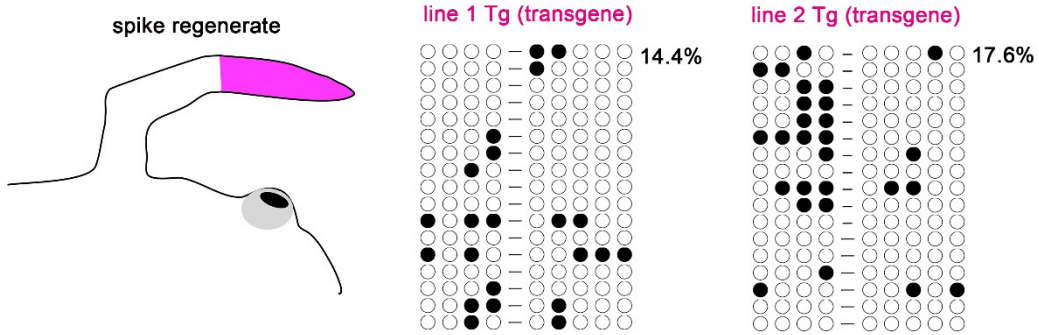



**Fig. S7. Methylation pattern of MFCS1 in a spike regenerate after limb regeneration of a froglet.** After limb blastemas of froglets completed spike regeneration, regenerate samples (pink) were obtained from line 1 and line 2 Tg frogs.

**Table S1. Rate of germline transmission and activity of MFCS1-GFP**

| G0 | N1 | N2 |
|---|---|---|
| | GFP+/ total | GFP+/ total |
| line 1 | 195/368 (53%) | 70/172 (41%) |
| line 2 | 79/180 (44%) | not applicable |

**Table S2. Supernumerary limb formation and ectopic GFP in rotated limb buds**

180° rotated limb buds

| Sample | No. of original digits | No. of supernumerary digits | No. of digits (total) | Is there ectopic GFP? | Figure |
|---|---|---|---|---|---|
| 1 | 5 | 2 | 7 | Yes | - |
| 2 | 5 | 2 | 7 | Yes | - |
| 3 | 5 | 2 | 7 | Yes | - |
| 4 | 5 | 2 | 7 | Yes | - |
| 5 | 5 | 4 | 9 | Yes | Fig. 2F, G |
| 6 | 5 | 2 | 7 | Yes | - |
| 7 | 5 | 1 | 6 | Yes | Fig. 2D, E |
| 8 | 5 | 0 | 5 | No | - |
| 9 | 5 | 0 | 5 | No | - |
| 10 | 5 | 0 | 5 | No | - |
| 11 | 5 | 2 | 7 | Yes | - |

0° rotated limb buds (controls)

| Sample | No. of original digits | No. of supernumerary digits | No. of digits (total) | Is there ectopic GFP? | Figure |
|---|---|---|---|---|---|
| 1 | 5 | 0 | 5 | No | Fig. 2B, C |
| 2 | 5 | 0 | 5 | No | - |
| 3 | 5 | 0 | 5 | No | - |
| 4 | 5 | 0 | 5 | No | - |
| 5 | 5 | 0 | 5 | No | - |
| 6 | 5 | 0 | 5 | No | - |
| 7 | 5 | 0 | 5 | No | - |
| 8 | 5 | 0 | 5 | No | - |
| 9 | 5 | 0 | 5 | No | - |
| 10 | 5 | 0 | 5 | No | - |
| 11 | 5 | 5 | 10 | Yes | - |
| 12 | 5 | 3 | 8 | Yes | - |

## Table S3. Primers for genomic- and RT-PCR

| name | Sequence (5’ -> 3’) | note |
|---|---|---|
| xtMFCS1-Fw | ACTGTAACCAGTGAAAATCATCAGTCATGAATATC | transgene |
| xtMFCS1-Rv | TGGCCTAGTTCATAGACATTAATTATTTTTCTATGGG | transgene |
| xtM1-Acc65I-Fw | ttggtaccTTCAAATGATGGATTTTCATATTATAA | Luc reporter |
| xtM1-XhoI-Rv | ggctcgaGACCATGAAGGCACCCTAATACT | Luc reporter |
| xlpS-XhoI-Fw | ggctcgAGTGACACCGTGGAAGCCTTTATTATC | Luc reporter |
| xlpS-NcoI-Rv | ggccatggtGTCCGAGCGAAGCCAATTACATCA | Luc reporter |
| Fgf2_cds_F1 | GCCATGGCTGCCAGCGGCATCACC | Gene Expression |
| Fgf2_cds_R1 | TCAGCTCTTAGCAGACATTGGAAG | Gene Expression |
| Fgf4_cds_F1 | CCGGGATGGCGAAACGCGGGCCGA | Gene Expression |
| Fgf4_cds_R1 | TCACAGTCTAGGAAGGAAGTGGGT | Gene Expression |
| Fgf8_cds_F1 | GACCCCGCTCGGGCTCTCAGTGCT | Gene Expression |
| Fgf8_cds_R1 | CTATCGGGGCTCCGGGGCCCAAGT | Gene Expression |
| Hoxd13-F | ATGAGCCGCTCGGGGACTTG | Gene Expression |
| Hoxd13-R | TCAGGAGACAGTGTCTTTGA | Gene Expression |
| MFCS1_BS_Fw | GTGTAATTAATTAGGTGGGyyAGGTGGAAA | Bisulfite sequencing |
| MFCS1_BS_Rv | TTAArCArCACTCCCTTAATCACTCATTTC | Bisulfite sequencing |
| M13F-pUC | GTTTTCCCAGTCACGAC | Bisulfite sequencing |
| M13R-pUC | CAGGAAACAGCTATGAC | Bisulfite sequencing |