

1 **Osteoclast-mediated resorption primes the skeleton for successful
2 integration during axolotl limb regeneration**

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29 **Keywords**

30 Axolotl, regeneration, skeleton, osteoclasts, integration

31 **ABSTRACT**

32

33 Early events during axolotl limb regeneration include an immune response and the formation of a wound
34 epithelium. These events are linked to a clearance of damaged tissue prior to blastema formation and
35 regeneration of the missing structures. Here, we report the resorption of calcified skeletal tissue as an
36 active, cell-driven and highly regulated event. This process, carried out by osteoclasts, is essential for a
37 successful integration of the newly formed skeleton. Indeed, the extent of resorption is directly
38 correlated with the integration efficiency. Moreover, we identified the wound epithelium as a major
39 regulator of skeletal resorption, likely creating a zone of influence in which signals involved in
40 recruitment/differentiation of osteoclasts are released. Finally, we reported a correlation between
41 resorption and blastema formation, particularly, a coordination of resorption with cartilage
42 condensation. In sum, our results identify resorption as a major event upon amputation, playing a critical
43 role in the overall process of skeletal regeneration.

44 INTRODUCTION

45

46 Urodele amphibians, such as the axolotl (*Ambystoma mexicanum*) are widely considered
47 prodigies among regenerative vertebrates. The ability to regenerate different body structures, especially
48 the limb, has driven years of scientific research aiming to understand the mechanisms underlying
49 regeneration. The axolotl limb is a complex structure, and its regeneration requires an intricate
50 choreography of all the cellular components. Beyond making new cells of the right type at the right
51 place, a successful regeneration requires a functional integration of those new cells with the pre-existing
52 tissue, a process that has not been widely studied. In particular, remains unknown how early processes
53 impact tissue integration.

54 In general, regeneration progression is marked by different overlapping phases, which lead to
55 the re-establishment of the missing limb (Sandoval-Guzmán and Currie, 2018). Two of the most critical
56 events are the formation of the wound epithelium (WE) and the blastema (Aztekin, 2021; Tanaka, 2016).
57 The WE is formed by migrating keratinocytes which close the wound in just a few hours (Hay and
58 Fischman, 1961; Repesh and Oberpriller, 1978). Importantly, the WE is characterized by the absence of
59 a basal lamina, which enhances the diffusion of important factors for regeneration (Neufeld and
60 Aulhouse, 1986; Repesh and Oberpriller, 1978). Indeed, the WE is a major regulator of the immune
61 response, tissue histolysis (Tsai et al., 2020), and blastema proliferation and patterning (Boilly and
62 Albert, 1990; Ghosh et al., 2008; Han et al., 2001). Notably, several works have demonstrated that the
63 WE is required for blastema formation and thus, regeneration (Mescher, 1976; Tassava and Garling,
64 1979; Thornton, 1957; Tsai et al., 2020).

65 The blastema is a heterogenous pool of progenitor cells arising from the various tissues at the
66 amputation plane (Kragl et al., 2009). Among the various limb components, the connective tissue (CT)
67 is a critical cell source for the blastema, supplying well over 40% of the cells within (Currie et al., 2016;
68 Dunis and Namenwirth, 1977; Gerber et al., 2018; Muneoka et al., 1986). Limb CT is a conglomerate
69 of different cell types which are found in tendons, skeleton, dermis and surrounding the skeleton (i.e.
70 periskeleton), muscle and blood vessels. A particular case is the skeleton, where cells embedded in the
71 skeletal matrices do not actively participate in regeneration (Currie et al., 2016; McCusker et al., 2016),
72 instead, dermal and periskeletal cells rebuild the new skeleton (Currie et al., 2016; Dunis and
73 Namenwirth, 1977; McCusker et al., 2016; Muneoka et al., 1986). Although the skeleton represents
74 more than 50% of the exposed surface upon amputation (Hutchison et al., 2007), it is unclear the role
75 the embedded cells play in the remodeling and integration of new tissue.

76 Undoubtedly, the skeletal system is essential for the limb, serving as a physical scaffold and
77 allowing locomotion. In mammals, the appendicular skeleton develops by endochondral ossification, a
78 process where a cartilage anlage is replaced by bone (Kozhemyakina et al., 2015). In axolotls, we
79 showed that the limb skeleton is progressively ossified with growth and age (Riquelme-Guzmán et al.,
80 2021), but retains a cartilage anlage even in the oldest specimen analyzed. Juvenile axolotls present a

81 cartilaginous skeleton composed of chondrocytes and perichondral cells. Around the time animals reach
82 sexual maturity, the cartilaginous skeleton is partly replaced by bone cells, adipocytes, and blood vessels
83 during ossification. Key players in this process are osteoclasts, a myeloid-derived population, which
84 mediates the degradation of the cartilage matrix prior to bone formation.

85 Osteoclasts are giant multinucleated cells with a specialized morphology adapted for skeletal
86 resorption (Cappariello et al., 2014). Besides their role in homeostasis, osteoclasts are recruited upon
87 bone injuries or trauma. The most studied case is fracture healing (Einhorn and Gerstenfeld, 2015);
88 however, in the context of regeneration, osteoclasts are recruited after fin amputation in zebrafish (Blum
89 and Begemann, 2015), and digit tip amputation in mouse (Fernando et al., 2011). In urodeles, evidence
90 of osteoclast-mediated resorption is scarce (Fischman and Hay, 1962; Tank et al., 1976). Nevertheless,
91 the presence of myeloid cells triggered by the amputation has been reported (Debuque et al., 2021; Leigh
92 et al., 2018; Rodgers et al., 2020), and the participation of macrophages was shown to be critical. When
93 macrophages were ablated, a complete halt in regeneration was reported (Godwin et al., 2013). Similar
94 results were observed upon mouse digit tip amputation, and a specific osteoclast inhibition resulted in
95 delayed bone resorption, wound closure and blastema formation; however, regeneration proceeded
96 (Simkin et al., 2017).

97 Immune cells play an important role in histolysis, which involves the degradation of the
98 extracellular matrix (ECM) in the vicinity of the amputation plane (Stocum, 2017), helping the
99 mobilization of progenitor cells (Thornton, 1938a, 1938b). Histolysis is characterized by the release of
100 proteolytic enzymes, essential for an efficient regeneration (Huang et al., 2021; Vinarsky et al., 2005;
101 Yang et al., 1999; Yang and Byant, 1994). Histolysis is additionally controlled by the WE (Vinarsky et
102 al., 2005), as shown by a major down-regulation of degrading enzymes upon the inhibition of WE
103 formation (Tsai et al., 2020). Similarly, macrophage ablation resulted in a down-regulation of matrix
104 metalloproteinases (MMPs) (Godwin et al., 2013).

105 Successful limb regeneration is achieved by a complete amalgamation of the regenerated
106 structures with the mature tissues, or tissue integration. Although the regenerated limb is often
107 considered a perfect replica of the pre-existing limb, in the last decade the fidelity of limb regeneration
108 has been addressed by a couple of works. For instance, abnormalities due to conspecific bites were
109 observed in 80% of larvae and 50% of adults (Thompson et al., 2014), or anomalies in over 50% of the
110 amputated animals, such as fractures at the level of amputation or constrictions of the skeletal elements
111 (Bothe et al., 2020). However, it is still unknown why such phenotypes are observed, and what entails
112 successful versus unsuccessful regeneration. In this regard, regeneration-specific signals in the stump
113 tissue could prime the limb and promote a successful integration of the newly formed structures. Indeed,
114 in the newt *Cynops pyrrhogaster*, structural changes in the ECM of the distal humerus can be observed
115 after an elbow joint amputation, demonstrating a correlation between ECM remodeling and proper joint
116 regeneration as well as integration to the mature tissue (Tsutsumi et al., 2015).

117 With all the aforementioned evidence, we sought to assess the significance of skeletal histolysis
118 for regeneration. We observed a rapid skeletal resorption which is carried out by osteoclasts, and we
119 provide evidence that this process is essential for tissue integration. Moreover, we propose a role for the
120 WE in resorption induction and a spatiotemporal coordination between resorption and blastema
121 formation. Overall, our work provides an in-depth assessment of how a remodeling process influences
122 the final outcome of regeneration using the axolotl limb.

123 **RESULTS**

124

125 **Skeletal elements are resorbed upon amputation**

126 To determine the changes in the skeleton upon amputation, we used the stable calcium-binding
127 dyes calcein and alizarin red. These dyes label mineralized cartilage in juvenile axolotls, allowing *in*
128 *vivo* imaging (Riquelme-Guzmán et al., 2021). Using 4-6 cm ST (snout-to-tail) axolotls, we amputated
129 the zeugopod at the distal end of the calcified tissue and imaged at different days post amputation (dpa)
130 (Fig. 1A). We observed a consistent reduction in the calcein⁺ tissue from 7 until 12 dpa. We quantified
131 the length of the calcified tissue in both zeugopodial elements and compared them to the initial length
132 at day 0 (Fig. 1B). Resorption initiated after 7 dpa and by 12 dpa, over 40% of the calcified radius and
133 60% of the calcified ulna were resorbed (length resorbed radius: $342.83 \pm 95.75 \mu\text{m}$; ulna: $770.67 \pm$
134 $94.34 \mu\text{m}$). We pooled five independent experiments and noticed an important variability between
135 assays (Fig. 1C, each color represents an assay). The median for radius resorption is 40% and for ulna
136 60%; however, in several cases the calcified tissue was completely resorbed in both elements. Although
137 an inter-assay variability was observed, intra-assay animals presented a consistent resorption ratio.

138 Digits are a simplified platform to perform *in vivo* imaging, therefore we assessed resorption by
139 amputating the distal end of the calcein⁺ tissue in the distal phalanx of the second digit (Fig. 1D). Similar
140 to the zeugopod, we quantified the calcein⁺ tissue length at different dpa and revealed a similar trend in
141 the resorptive dynamics: resorption starting after 7 dpa and receding by 13 dpa (Fig. 1E), vanishing over
142 50% of the calcified tissue length ($320.43 \pm 113.56 \mu\text{m}$). In sum, we report resorption to be a process
143 that occurs upon amputation of different calcified skeletal elements in the axolotl limb.

144 Finally, we collected limbs at 9 and 15 dpa and stained them with alcian blue (Fig. 1F). At 9
145 dpa, we observed resorption in both radius and ulna. Remarkably, we occasionally observed a break in
146 the ulna (Fig. 1F arrowhead) that sometimes led to the extrusion of the skeletal fragment through the
147 epidermis. This skeletal shedding was observed both in digit and limb amputations. At 15 dpa, resorption
148 was finished and the condensation of the new skeleton could be observed.

149

150 **Osteoclasts are identified during skeletal resorption**

151 Osteoclasts are specialized multinucleated cells responsible for skeletal resorption (Charles and
152 Aliprantis, 2014). Despite their critical role in skeletal biology, osteoclasts in salamander regeneration
153 have only been reported on the basis of morphology during salamander regeneration (Fischman and
154 Hay, 1962; Nguyen et al., 2017; Tank et al., 1976). Therefore, we sought to identify osteoclasts during
155 resorption using various molecular markers.

156 Several enzymes, such as cathepsin K (CTSK) and the tartrate-resistant acid phosphatase
157 (TRAP) (Cappariello et al., 2014), are released by osteoclasts and are used as identifying markers. Using
158 sections from zeugopodial amputations, we performed immunofluorescence using an anti-CTSK
159 antibody (Fig. 2A) and TRAP enzymatic staining (Fig. 2B). CTSK⁺ cells were identified in sections at

160 8 dpa adjacent or inside the calcein⁺ skeleton. Similarly, TRAP⁺ cells were identified at 9 dpa. Next, to
161 correlate osteoclast recruitment with resorption timing, we performed RT-qPCR at different dpa using
162 specific primers for *Trap*, *Ctsk* and *Dcstamp* (dendritic cell-specific transmembrane protein, involved
163 in osteoclast multinucleation). The RNA relative content for the three markers behaved similarly: a
164 sharp increase was observed, reaching a peak at 9 dpa before rapidly decrease to almost basal levels at
165 15 dpa (Fig. 2C).

166 To assess osteoclast spatiotemporal dynamics *in vivo*, we developed a *Ctsk:mRuby3* and
167 *Ctsk:eGFP* transgenic lines, which express the fluorescent protein *mRuby3* or *eGFP* under the control
168 of *Ctsk* promoter from zebrafish. Using *Ctsk:mRuby3* animals, we followed resorption in digits with
169 confocal microscopy (Fig. 2D). At 0 dpa, the tissue was devoid of *mRuby3*⁺ cells. At 9 dpa,
170 mononuclear-like cells were observed in the periphery of the calcified phalanx. These cells increased in
171 numbers and size at 11 dpa (white arrowheads). A break in the phalanx (black arrowhead) was seen at
172 this timepoint. At 13 dpa, most of the phalanx was resorbed and *mRuby3*⁺ cells were scattered
173 throughout the sample. A giant multinucleated cell was observed next to the calcified tissue (asterisk
174 Fig. 2D). Finally, between 13 and 15 dpa, resorption was completed and *mRuby3*⁺ cells vacated the
175 space, some showing signs of apoptotic puncta. Although most osteoclasts were multinucleated, we also
176 observed mononuclear cells. Whether this indicates differences in osteoclast biology between axolotls
177 and other model organisms, is not yet determined. Nevertheless, by utilizing different approaches, we
178 demonstrated the presence and participation of osteoclasts in the regeneration-induced resorption.

179

180 **Zoledronic acid treatment inhibits osteoclast-mediated skeletal resorption**

181 To assess the effect of osteoclast inhibition on regeneration, we treated animals with the
182 osteoclast inhibitor zoledronic acid (zol). Zol is a potent bisphosphonate, used in the treatment of
183 osteoporosis. It is internalized by osteoclasts, preventing protein prenylation and consequently their
184 intracellular localization and function (Dhillon, 2016), which could lead to apoptosis (Clézardin, 2013).
185 By serial intraperitoneal injections of 200 µg/kg of zol every 3 days, we evaluated the effect of osteoclast
186 inhibition by imaging the length of the skeletal elements at different dpa. Zol treatment inhibited
187 resorption as seen at 12 dpa (Fig. 3A), since most of the calcified tissue remained intact when compared
188 to the untreated control and vehicle. Quantification of both radius and ulna lengths at different dpa
189 revealed a significant difference between the radius or ulna in zol-treated animals compared to the
190 controls at 11, 12 and 15 dpa (Fig. 3B).

191 Furthermore, we measured the relative RNA content of *Ctsk*, *Trap* and *Dcstamp* at 9 dpa in each
192 condition. No significant difference was observed for the three markers (Fig. 3C), although the mean
193 for zol-treated samples was smaller in each case. Our results suggested that zol treatment mainly results
194 in a consistent inhibition of osteoclast function. Consequently, we performed *in vivo* imaging of digit
195 regeneration upon zol treatment in the *Ctsk:eGFP* transgenic line. When resorption was inhibited by zol
196 treatment, we observed a reduction in the number of *eGFP*⁺ cells (Fig. 3D). Although present, these cells

197 did not seem to resorb the calcified tissue. Therefore, zol treatment inhibits osteoclast-mediated
198 resorption, but it does not result in their complete ablation.

199

200 **Skeletal resorption is necessary for a successful integration of the regenerated structure**

201 To assess the importance of resorption, we followed the zol-treated animals until 45 dpa. At this
202 stage, limbs are fully formed but they have not reached yet full size (Tank et al., 1976). Looking at the
203 gross morphology, resorption inhibition did not halt regeneration, as zol-treated animals were able to
204 form a new limb. We assessed integration by sequentially staining with calcium binding dyes of different
205 color, we distinguished the original calcification (calcein⁺) from the calcification of regenerated skeletal
206 elements (alizarin red⁺) (Fig. 4A, left panel). In contralateral limbs, alizarin red staining showed new
207 calcification from the calcein staining at 0 dpa. Comparatively, we observed no calcein⁺ tissue in the
208 untreated animals, indicating a full resorption of the calcified tissue in the radius and ulna. The alizarin
209 red⁺ region demonstrated regeneration of the skeleton. In zol-treated animals, at least half of the calcified
210 region was calcein⁺/alizarin red⁺, confirming resorption inhibition. Interestingly, we observed a faulty
211 morphology in the radius from the zol-treated animal (arrowhead Fig. 4A, left panel). To gain a better
212 insight into the morphology of the regenerated zeugopod, we collected those limbs and stained them
213 with alcian blue (Fig. 4A, right panel). The zol-treated limb showed a clear failure in the integration of
214 the newly formed cartilage, especially in the radius. The new tissue lacked a seamless connection to the
215 stump part, presenting an angulated morphology (black arrowhead). In the ulna, heterotopic cartilage
216 formation was seen (asterisk). Surprisingly, the skeletal elements of untreated regenerating animals also
217 showed imperfect morphology, even though the calcified areas were fully resorbed. Both radius and
218 ulna were restored as one complete unit, but with an irregular interphase between the stump and
219 regenerated part, observed as a narrowing in the mid-diaphysis (black arrowhead, Fig. 4A).

220 Among the control and zol-treated limbs, we found different rates of resorption (Fig. 4B). A
221 correlation between resorption rate and integration efficiency could be observed, particularly for the
222 radius. In a zol-treated animal, with null resorption in the radius (R:0), the distal end of the stump part
223 and the proximal end of the regenerated skeleton failed to meet. The regenerated skeleton formed at an
224 adjacent plane, therefore lacking continuity with the pre-existing skeleton. Moreover, a second
225 condensation zone was seen, as cartilage also formed distal to the un-resorbed tissue.

226 To consistently quantify the integration success, we analyzed only animals undergoing normal,
227 undisturbed regeneration. This way, with less severe phenotypes and the skeletal elements regenerating
228 as one unit, it was simpler to correlate resorption to integration. The angle of the regenerated skeletal
229 elements to the stump skeletal element was measured. In 18/22 untreated animals, their radii were not
230 fully resorbed and presented different degrees of angulation, between 135° to 165°, at the stump-
231 regenerated interphase (Fig. 4C). These results show that even in the best-case scenario, limb
232 regeneration in the axolotl can lead to an imperfect outcome.

233 We assessed whether the faulty integration was resolved at later stages. We collected limbs at
234 90 dpa and stained them with alcian blue/alizarin red. In 6/6 limbs screened, in which the resorption rate
235 was over 50% for both elements, we could still observe a faulty integration of both radius and ulna
236 (arrowheads, Fig. 4D). This imperfect integration was identified by an angulation at the stump-
237 regenerated interphase, similar to what we reported at 45 dpa.

238 As resorption has a clear impact on skeletal integration during regeneration, we sought to
239 analyze the ECM organization and its changes at the stump-regenerated interphase using quantitative
240 polarization microscopy (LC-PolScope) (Oldenbourg, 1996). We used limb sections from normally
241 regenerated animals for our analysis. By looking at the ECM organization (retardance image), we
242 observed a clear difference in the hypertrophic zone (HZ) of regenerated ulnas when compared to the
243 contralateral limb (arrowhead, Fig. 4E). We believe that this region in the HZ corresponded to the stump-
244 regenerated interphase. Next, using the retardance image, we created a digital mask that allowed us to
245 quantify the orientation of the ECM components using the slow axis orientation image (Fig. 4E, lower
246 panels). We defined two regions, the HZ, where the interphase is found, and the resting zone (RZ), a
247 control region proximal to the amputation plane. We generated a histogram representing the angle
248 distribution in each zone. We observed that the HZ in the contralateral ulnas presented a parallel
249 organization of the ECM respect to the proximo-distal axis, while the regenerated presented a shift in
250 the organization, with the ECM fibers arranged perpendicularly. The RZ remained unchanged in both
251 samples sets (Fig. 4F). This result shows that the regenerated ECM does not recapitulate the original
252 structure, and supports the idea that skeletal regeneration is not completely efficient in the axolotl.

253 Altogether, these results show the importance of resorption during skeletal regeneration and
254 requirement for integration of the regenerated tissue. However, these results also highlight that even in
255 normal conditions, the regenerated skeleton does not recapitulate the smooth structure seen pre-
256 amputation.

257

258 The wound epithelium is involved in resorption induction

259 A previous report showed that the WE is critical for inflammation and tissue histolysis (Tsai et
260 al., 2020). When the WE formation was prevented by mechanically closing the wound with stump tissue,
261 in a so-called full skin flap surgery (FSF), *Ctsk* expression was absent at 5 dpa compared to control.
262 This suggests potential defects in skeletal resorption and a role of the WE in its induction. Therefore,
263 we sought to evaluate the role of the WE in skeletal resorption.

264 Given the technical difficulty of this surgical procedure, we used 14 cm ST animals, similar to
265 the previous reported model (Tsai et al., 2020). We amputated the limbs prior to FSF surgery, and
266 followed them until 15 dpa. Using *in vivo* imaging, we observed an inhibition in resorption in FSF
267 samples by comparing calcified tissue length to the control limb (arrowhead Fig. 5A). Next, we collected
268 the limbs and performed alcian blue/alizarin red staining. In 7 out 9 control samples, we observed a clear
269 degradation in the distal end of the skeletal elements (black arrowhead Fig. 5B), while no or limited

270 resorption was observed in the FSF limbs. To further confirm resorption inhibition, we collected limbs
271 at 9 dpa and performed ISH for *Ctsk*. We saw a significant reduction of the *Ctsk* staining in FSF sections
272 (Fig. 5C), suggesting the WE plays a role in the recruitment or differentiation of osteoclasts.

273 By using 14 cm ST animals, we demonstrate that resorption occurs also when skeletal elements
274 in the limb are undergoing ossification. Limbs of older animals are opaquer and becomes harder to
275 quantify the length of the calcified tissue, thus, we performed micro-CT scans in limbs of animals 16
276 cm ST (Fig. supplement 1). We confirmed a significant resorption of ossified elements in a slightly
277 extended, but conserved time window than in small animals.

278 To evaluate whether the WE position might determine the region of resorption initiation, we
279 spatially correlated resorption and the WE. We performed WISH for *Krt17*, which labels cells in the
280 basal layers of the WE (Leigh et al., 2018). We observed a clear labelling of the WE from 1 to 7 dpa
281 (Fig. 5D). At 1 and 5 dpa, we observed that at least 1/3 of the skeletal elements (yellow dashed lines)
282 were covered by the WE, which could account for over 50% of the tissue that will be resorbed.
283 Morphologically, the WE is characterized by the absence of a basal lamina (Neufeld and Aulhouse,
284 1986; Repesh and Oberpriller, 1978; Tsai et al., 2020), hence we used this feature to correlate the WE
285 and resorption. We collected and sectioned limbs at 1, 5 and 7 dpa, and performed Masson's trichrome
286 staining. The lack of a basal lamina was observed by the absence of collagen staining in blue (yellow
287 arrowheads, Fig. 5E). Osteoclasts could be identified by their multiple nuclei and morphology. As
288 expected, we did not observe any osteoclast at 1dpa. At 5 dpa, we identified the first infiltration of the
289 skeletal elements, albeit a small number of osteoclasts (white arrowheads, Fig. 5E, F). Finally, we saw
290 pronounced infiltration at 7 dpa, including the presence of osteoclasts (white arrowheads, Fig. 5E, G).
291 Notably, most of these cells were located in the proximity of the WE. To evaluate the location of the
292 osteoclasts, we defined a region by drawing a line between the edges of the WE in the sections stained
293 and we mapped the position of each osteoclast at 7dpa (Fig. 5H). Most of the osteoclasts were located
294 in the region covered by the WE, i.e. in the distal part of the skeletal elements. In the more proximal
295 regions of the zeugopod, we did not observe any osteoclasts.

296 In sum, our data suggest that the WE plays a role in both osteoclasts recruitment and/or
297 differentiation, as well as in generating a zone of influence which determines resorption initiation in the
298 distal regions of the skeletal elements.

299

300 **Identification of candidates involved in osteoclast recruitment and/or differentiation**

301 To identify possible candidates involved in osteoclast recruitment and/or differentiation, we
302 curated a published RNA-seq dataset in which FSF surgery was performed (Tsai et al., 2020). In that
303 work, three different populations from blastemas at 5 dpa were isolated: dividing cells (4N), non-
304 dividing cells (2N) and epithelial cells (EP). We first checked which fraction was enriched for transcripts
305 associated with osteoclast function at 5 dpa. As expected, non-dividing cells (2N) were enriched for
306 osteoclasts genes (*Trap*, *Traf6*, *Rank*, *Ocstp*, *Nfatc1*, *Dcstamp*, *Ctsk*, *Csfr1*) (Fig. 6A). Moreover, in the

307 2N fraction at 5 dpa, most of those transcripts were up-regulated compared to day 0, and down-regulated
308 in FSF limbs at 5 dpa (Fig. 6B). This analysis supports our previous results, in which we observed an
309 inhibition of resorption when the WE formation was prevented.

310 Next, we evaluated which transcripts were significantly down-regulated in the EP fraction of
311 FSF limbs compared to a control limb at 5 dpa (supplementary information Tsai et al., 2020). We found
312 several transcripts associated with osteoclast recruitment and/or differentiation (Fig. 6C). From these
313 candidates, previous work reports a role for *Ccl4* (Xuan et al., 2017), *Sphk1* (Baker et al., 2010; Ishii et
314 al., 2009; Ryu et al., 2006) and *Mdka* (Maruyama et al., 2004) in osteoclastogenesis. Moreover, these
315 three transcripts were up-regulated at 5 dpa compared to 0 dpa, suggesting their participation in
316 regeneration (red arrows, Fig. 6C). Finally, we confirmed that most of the candidate transcripts shown
317 in Fig. 6C were expressed in the EP fraction (Fig. 6D), including *Sphk1* and *Ccl4*. One of the exceptions
318 was *Mdka*, whose levels were more prominent in the 4N fraction; however, it was recently shown that
319 *Mdka* plays a critical role in WE development and inflammation control during the earlier stages of
320 regeneration (Tsai et al., 2020). This analysis provides evidence that factors associated with
321 osteoclastogenesis and osteoclast recruitment, normally produced by the WE, are down regulated when
322 WE formation is inhibited. Correspondingly, osteoclast transcripts are down-regulated in blastema cells.
323

324 **Skeletal resorption and blastema formation are spatially and temporally correlated**

325 Blastema formation is considered an accumulation of cells at the amputation plane. However,
326 taking resorption into consideration, those cells are initially accumulating (or reprogramming) more
327 proximal to the amputation plane. Here, we showed that resorption can reach up to 100% of the calcified
328 tissue, and hence the accumulation of progenitor cells might occur up to 1 mm behind the amputation
329 plane. With this in mind, we sought to assess the blastema specification in the context of skeletal
330 resorption.

331 First, we measured the blastema surface in images taken at 15 dpa, when resorption is completed
332 and blastema already formed in zol-treated animals. We considered the distal end of the skeletal
333 elements as the starting point of blastema, as it was proposed that progenitor cells accumulate distal to
334 the end of the skeleton (Tank et al., 1976). As shown in Fig. 7A, we found a significant decrease in
335 blastema area in zol-treated animals (yellow dashed line). To efficiently analyze the blastema position
336 during resorption, we used molecular markers. First, we performed whole mount EdU staining at
337 different dpa (Fig. 7B). Similar to previous reports, in an intact limb, EdU⁺ cells are less than 0.5% of
338 the total cells (Johnson et al., 2018). At 7 dpa, we observed EdU⁺ cells behind the amputation plane,
339 spanning over more than 500 μ m. These cells were located where we expected to observe resorption.
340 Interestingly, several EdU⁺ cells were located in the periskeleton (arrowheads), which could account for
341 cells contributing to skeletal regeneration (Currie et al., 2016; McCusker et al., 2016). These cells were
342 found along most of the skeletal element length. At 10 dpa, when resorption is occurring, we observed
343 a more defined blastema (white arrowhead), which contained EdU⁺ cells. Similar to 7 dpa, a significant

344 proportion of those EdU⁺ cells were located next to the resorbing skeleton. Finally, at 15 dpa, we
345 observed an evident reduction in the skeletal length (yellow arrowheads) and a defined blastema distal
346 to those skeletal elements (white dashed line). At this point, very few EdU⁺ cells were found next to the
347 skeleton.

348 Although proliferation is mainly observed where blastema is forming, distal migration of EdU⁺
349 after division is also expected. The site of blastema formation was further assessed using a blastema
350 marker in intact and 7 dpa limbs. We chose *Kazald1*, since it was shown to play a critical role in blastema
351 formation (Bryant et al., 2017). Similar to the EdU⁺ labeling, we observed that *Kazald1*⁺ cells were
352 located behind the amputation plane, surrounding the distal ends of the skeletal elements (dashed lines),
353 in a zone where resorption is very likely to occur (Fig. 7C). These results suggest that blastema formation
354 occurs in the same region and at the same time as resorption, and that using the skeletal element as a
355 boundary for blastema identity, may provide an incomplete view of the course of regeneration.

356 Finally, to assess the cellular dynamics of skeletal resorption/formation, we used a *Sox9:Sox9-*
357 *T2a-mCherry*^{ns} transgenic line in conjunction with calcein staining to follow both processes *in vivo*.
358 We performed amputation of the distal phalanx and followed the same amputated digit at different dpa
359 (Fig. 7D). At 0 dpa, no mCherry⁺ cells were found outside the calcified tissue. At 9 dpa, we observed a
360 break in the calcified phalanx (arrowhead) which shows a place where resorption was initiated. At 11
361 dpa, we observed a disorganized aggrupation of mCherry⁺ cells distal to the resorbed calcified tissue
362 (white arrowhead). Those cells might represent the initial condensation of the regenerating cartilage.
363 Interestingly, at 13 dpa, resorption continued (yellow arrowhead) and we saw an increase in the number
364 and density of mCherry⁺ cells. Finally, at 15 dpa, resorption was finished and the condensation of
365 mCherry⁺ cells in the new phalanx presented defined pattern. The condensation observed from 11 dpa,
366 occurred behind the amputation plane, supporting our previous results. Moreover, these results show
367 that resorption and skeletal regeneration are overlapping processes.

368 **DISCUSSION**

369

370 Axolotl limb regeneration is an intricate multi-step process that requires the fine tuning of
371 events such as wound closure, tissue repair, progenitor cells recruitment and the re-establishment of the
372 functional unit. Although extensive work has been done to understand the cellular dynamics of blastema
373 formation, other processes such as tissue histolysis, the immune response and tissue integration have yet
374 to be fully understood.

375 Here, we report that upon digit and zeugopod amputation, significant skeletal resorption occurs
376 which is carried out by an osteoclast population and can result in the resorption of 100% of the calcified
377 matrix. Skeletal resorption is observed in young animals with cartilaginous limbs as well as young adult
378 animals with ossified bones. Upon inhibition of resorption, we observed a clear failure in the integration
379 of the regenerated zeugopodial elements and, interestingly, this failure can also occur in untreated
380 animals undergoing regeneration. Moreover, we present strong evidence supporting the role of the WE
381 in resorption induction. Finally, we observed a spatial correlation between resorption progression and
382 blastema formation. Particularly, we found that the condensation of new cartilage started before the
383 resorative process is completed.

384 Similar to salamanders, mouse digit tip regeneration progresses with early histolysis and
385 blastema formation. An extensive resorption of the phalanx can lead to bone volume reduction of almost
386 50% of its original size (Fernando et al., 2011). When osteoclasts are inhibited, regeneration is not
387 compromised (Simkin et al., 2017) and when wound closure is induced earlier, resorption is delayed
388 and regeneration progresses (Simkin et al., 2015). This suggest than in mouse digit tip resorption
389 facilitates blastema formation. Moreover, it has been shown that both periosteal and endosteal cells are
390 responsible for regeneration of the phalanx upon amputation in mice (Dawson et al., 2018), suggesting
391 that bone resorption may be required for mobilization of a pool of progenitor cells. Although bone
392 resorption is not required for regeneration to progress, this histolytic process is indeed an important
393 event for the efficient regeneration of the mouse digit tip. In contrast to mouse, axolotl skeletal elements
394 do not mobilize progenitors to the blastema and wound closure occurs within hours upon amputation,
395 even when skeletal structures protrude at the surface. An important similarity to our findings in axolotl
396 is the time frame when resorption occurs (7-15 dpa). This suggest that the unique fast activation and
397 clearance of osteoclasts is particular to regeneration.

398

399 **Resorption efficiency defines skeletal integration success**

400 In this work, osteoclast inhibition resulted in a clear failure in tissue integration. This phenotype
401 often presented as an angulation of the radius, heterotopic cartilage formation in the ulna, or a complete
402 separation between the mature and the regenerated structures. In general, we observed a higher rate of
403 resorption for the ulna than for the radius, which could account for the more striking integration
404 phenotypes reported for the radius.

405 Our experiments revealed a gradient of integration phenotypes correlated with the amount of
406 tissue resorbed: the more resorption, the better the integration. Strikingly, in animals undergoing normal
407 regeneration we often observed faulty integration phenotypes in mineralized skeleton, as seen by
408 angulations at the stump-regenerated interphase. A recent report, showing abnormally regenerated limbs
409 in almost 50% of the animals, showed some similar phenotypes to the ones presented here, i.e. a
410 narrowing in the diaphysis and some heterotopic cartilage formation (Bothe et al., 2020). Using
411 polarization microscopy, we presented evidence of an ECM disorganization in the interphase region
412 where both stump and regenerated tissue are connected. Finally, we showed a prevalence of these
413 phenotypes at 90 dpa, proving that they are not resolved after regeneration has been completed.

414 Remarkably, we observed a high variability in the amount of calcified tissue resorbed in
415 different animals, ranging from 25 to 100% for radius and ulna, being the inter-assay variability higher
416 than the intra-assay. The source of this variability could be an environmental factor (e.g. water
417 temperature), but it highlights the different outcomes that skeletal regeneration can produce. Indeed, in
418 some cases, resorption involved a sequential degradation of the skeletal tissue, and at other times, it
419 involved the break and shedding of a skeleton piece, which has been observed in mouse digit tip
420 (Fernando et al., 2011). In contrast to mouse, skeletal shedding in the axolotl is not associated with
421 wound re-epithelialization, as this occurs after wound closure.

422 Our results highlight the misconception that axolotl limb regeneration recapitulates the pre-
423 existing morphology with high fidelity. This report reveals that a faulty skeletal regeneration is a rather
424 common outcome in the axolotl limb, and correlates resorption efficiency with successful skeletal
425 integration.

426

427 **Wound epithelium, resorption and blastema**

428 The WE is a critical structure for the progression of regeneration, regulating process such as
429 ECM remodeling, tissue histolysis, proliferation and inflammation (Tsai et al., 2020). Here, by blocking
430 the formation of the WE, we underscore its role in the initiation of resorption. Moreover, we analyzed
431 available WE RNA-seq data and found several factors known to influence osteoclast progenitor
432 migration and/or differentiation. Among those candidates, *Sphk1*, *Ccl4* and *Mdka* are up-regulated in
433 the epithelial fraction during regeneration and have been linked to osteoclast biology. Indeed, S1P,
434 which is phosphorylated by the sphingosine kinase (SPHK), has been shown to have a role in both bone
435 resorption and bone formation (Ishii et al., 2009; Pederson et al., 2008; Ryu et al., 2006), while CCL4
436 and MDKA have been connected with osteoclast progenitors recruitment (Maruyama et al., 2004; Xuan
437 et al., 2017). Future studies to understand how these factors are regulated in osteoclast-mediated
438 resorption during regeneration will be needed.

439 We hypothesized that the connection between the WE and skeletal resorption could be mediated
440 by a zone of influence determined by the WE position. Our results show that resorption starts distally,
441 enclosed by the WE boundaries, supporting this idea. Although the WE may not be the only source of

442 factors inducing osteoclast progenitor migration and differentiation, it probably is a general source of
443 chemokines inducing the recruitment of RANK⁺ myeloid progenitors. We hypothesize that those
444 myeloid cells will then recognize factors secreted by the skeletal elements that promote osteoclast
445 differentiation. Indeed, the main source of RANKL in mammals are both hypertrophic chondrocytes
446 and osteocytes (Xiong et al., 2011).

447 In this work, we also provide evidence supporting a spatiotemporal correlation between skeletal
448 resorption and blastema formation. The WE produces signals involved in blastema proliferation and
449 patterning (Boilly and Albert, 1990; Ghosh et al., 2008; Han et al., 2001; Tsai et al., 2020), and those
450 signals could be acting in the same zone of influence. Moreover, since the skeletal elements are
451 structural supports of the limb, the resorption of the hard matrix might cause a collapse of the soft tissue
452 towards the proximal region, and thus a formation of the blastema behind the amputation plane. Indeed,
453 we observed EdU⁺ cells and *Kazald1*⁺ cells in the surroundings of the skeletal elements shortly before
454 the start of resorption, and condensation of skeletal progenitors distal to the resorbed tissue and under
455 the amputation plane.

456 Historically, the amputation plane has been conceptualized as a fixed position in the limb which
457 determines the beginning of the blastema. However, the majority of the amputation experiments are
458 performed by trimming the skeletal elements because this ensures consistency on the formation of a
459 blastema. In this surgical procedure, the skin of the amputated limb is retracted and the extending
460 skeletal elements are re-amputated. Trimming results in a faster WE and blastema formation; however,
461 this procedure might cause the erroneous perception of a fixed amputation plane. Comparatively, in the
462 case of the mouse digit tip, in which bone resorption occurs, a regeneration plane has been identified as
463 more proximal than the amputation plane. (Seifert and Muneoka, 2018). This study has implications for
464 demarcating the blastema, the mature cell source and the dynamic interphase created by nascent,
465 migrating cells and a stream of morphogens.

466

467 Future perspectives

468 There are still unresolved questions regarding the osteoclast population here described. First,
469 what is the origin of this population during regeneration? Upon amputation, a peak of myeloid
470 chemotactic molecules was reported at 1 dpa, followed by an infiltration of myeloid cells and
471 macrophages (Godwin et al., 2013). This could suggest that osteoclast progenitors are recruited to the
472 amputation plane, but it does not rule out the participation of resident progenitors in the neighboring
473 tissues. Second, what is the fate of osteoclasts after resorption? We show here that this population
474 eventually disappears and even shows some signs of apoptosis, but whether all undergo apoptosis or if
475 some will recirculate, remains unknown. Current understanding of osteoclast biology suggest that they
476 are short-lived cells, which undergo apoptosis after ca. 2 weeks (Manolagas, 2000). However, recent
477 works have shown that osteoclasts can be longer lived (Jacome-Galarza et al., 2019) or be recycled via
478 a cell type named osteomorphs (McDonald et al., 2021). These studies underline the need to continue

479 investigating osteoclast biology *in vivo*, and particularly in this rapidly-triggered response to
480 regeneration. The axolotl limb presents a new paradigm in which osteoclast function can be assessed,
481 and thus the development of new transgenic lines to label myeloid progenitors and to indelibly label
482 osteoclast will provide a mean to resolve the aforementioned questions.

483 In addition, the concomitant resorption and regeneration need to be further explored. It is
484 known that histolysis helps with the mobilization of progenitor cells in salamanders (Thornton, 1938b)
485 and in mouse (Dawson et al., 2018), but how osteoclast-mediated resorption could be influencing
486 cartilage condensation in the context of axolotl regeneration remains to be studied. Specifically, how
487 the cell differentiation and migration is orchestrated with respect to resorption is unclear. Of particular
488 interest are periskeletal cells migrating from the periphery of the skeletal element towards the blastema,
489 and contributing to the formation of the proximal skeleton. The cell source zone, i.e. a zone where
490 blastema cells are recruited, has been roughly defined to be 500 μm from the amputation plane.
491 However, resorption was not analyzed in that study (Currie et al., 2016). It is unclear how resorption
492 and the detachment and migration of periskeletal cells are coordinated, or if the source of periskeletal
493 cells correspond to a region not resorbed (e.g. proximal to the calcified tissue). Previous works have
494 demonstrated the interaction between osteoclast and osteoblasts *in vivo* (Furuya et al., 2018; Ikebuchi
495 et al., 2018), and the *in vivo* assessment of this in the context of skeletal regeneration would be necessary.

496 Finally, we need to consider the context in which resorption is occurring since different cell
497 types are found in the same skeletal element along the proximo-distal axis, and this could influence the
498 outcome of resorption in skeletal regeneration (Riquelme-Guzmán et al., 2021).

499

500 **Concluding remarks**

501 This work presents a systematic assessment of the timing, extent and consequences of skeletal
502 resorption. We show that the skeleton undergoes a massive and rapid histolytic event which is essential
503 for a successful integration of the regenerated structure. This process, which is carried out by osteoclasts,
504 is influenced by the formation of the WE and is correlated with the spatial position of the early blastema.
505 Furthermore, we present proof that the axolotl limb regeneration is not perfect and it often leads to
506 abnormal skeletal phenotypes. We consider that resorption is playing a key role in skeletal regeneration
507 and its implications for regeneration needs to be further explored, particularly its coordination with cell
508 migration and condensation of the new skeleton.

509 **MATERIALS AND METHODS**

510

511 **Axolotl husbandry and transgenesis**

512 Axolotls (*Ambystoma mexicanum*) were maintained at the CRTD axolotl facility and at Harvard
513 University. All procedures were performed according to the Animal Ethics Committee of the State of
514 Saxony, Germany, and the Institutional Animal Care and Use Committee (IACUC) Guidelines at
515 Harvard University (Protocol 11-32). Animals used were selected by its size (snout to tail = ST). Most
516 experiments were done using animals 4-6 cm ST, unless stated otherwise. We performed experiments
517 using white axolotls (*d/d*). In addition, we utilized transgenic lines shown in table I.

518

519 Table I: Transgenic lines used in this work.

| Name | Here referred as | Reference |
|---|---------------------------------|--------------------------------|
| C-Ti ^{U+} (<i>Sox9:Sox9-T2a-mCherrynls</i>) ^{ETNKA} | <i>Sox9:Sox9-T2a-mCherrynls</i> | (Riquelme-Guzmán et al., 2021) |
| tgTol2(<i>Drer.Ctsk:mRuby3</i>) ^{tsg} | <i>Ctsk:mRuby3</i> | This work |
| tgTol2(<i>Drer.Ctsk:eGFP</i>) ^{tsg} | <i>Ctsk:eGFP</i> | This work |

520

521 To generate the *Ctsk:mRuby3* or *Ctsk:eGFP* transgenic lines, a plasmid containing 4 kb of *Ctsk*
522 promoter from zebrafish together with *Tol2* sequences was used (kind gift from Knopf Lab at CRTD
523 and Gilbert Weidinger at Ulm University). The *mRuby3* or *eGFP* coding region was cloned 3' from the
524 promoter. For ligation, plasmid restriction was performed using the *FseI* and *XhoI* restriction enzymes
525 (#R0588S, #R0146S respectively; New England BioLabs, Frankfurt am Main, Germany). Fertilized
526 embryos from *d/d* axolotls were injected with the *Ctsk:mRuby3* or *Ctsk:eGFP* vector and *Tol2* mRNA
527 as previously described (Khattak et al., 2014). F0 animals were selected and grown in our colony until
528 sexual maturity. For experiments, F0 were crossed with a *d/d* axolotl, and F1 animals were used.

529

530 **Experimental procedures in axolotls**

531 *In vivo* skeletal staining was performed using calcein (#C0875, Sigma-Aldrich, Darmstadt,
532 Germany) or alizarin red (#A5533, Sigma-Aldrich) A 0.1% solution was prepared for either dye with
533 swimming water. Axolotls were submerged in solution for 5 - 10 minutes in the dark. After staining,
534 animals were transferred to a tank with clean swimming water, which was changed as many times until
535 water was not stained. Amputations were performed either 10 minutes after staining or the next day for
536 better visualization.

537 For amputations, animals were anesthetized with 0.01% benzocaine solution. All amputations
538 were performed at the distal end of the calcified diaphysis using an Olympus SZX16 stereomicroscope.
539 After surgical procedure, animals were covered with a wet tissue (with benzocaine) and allowed to

540 recover for 10 minutes prior to be transferred back to swimming water. The full skin flat surgery (FSF)
541 was performed as described in (Tsai, 2020; Tsai et al., 2020).

542 Zoledronic acid (#SML0223, Sigma-Aldrich) treatment and EdU (#C10337, Invitrogen,
543 Darmstadt, Germany) labelling were done by intraperitoneal injections in anesthetized axolotls. 200
544 µg/kg of zoledronic acid were injected every 3 days (stock 1 mg/mL in APBS (80% PBS)). 10 µg/g of
545 EdU were injected 4 hours prior to tissue collection (stock 2.5 mg/mL in DMSO). Injection volume was
546 adjusted to 10 µL with APBS. After injections, animals were kept covered with a wet paper for 10
547 minutes before returning them into the water tank.

548 *In vivo* imaging was performed in anesthetized animals. For stereoscope imaging, animals were
549 placed in a 100 mm petri dish and limb was positioned accordingly. An Olympus SZX16 stereoscope
550 microscope (objective: SDF Plapo 1xPF) was used. For confocal imaging, animals were place in a glass
551 bottom dish (ø: 50/40 mm, #HBSB-5040, Willco Wells, Amsterdam, The Netherlands). A wet tissue
552 with benzocaine was laid on top of the animal to avoid it to dry, and a silica block was laid on top of the
553 hand to flatten it and improve light penetrance. A Zeiss confocal laser scanning microscope LSM780
554 (objectives: Plan apochromat 10x/0.45 or Plan-apochromat 20x/0.8) was used.

555 For tissue collection, animals were anesthetized prior to collection. After it, animals were
556 euthanized by exposing them to lethal anesthesia (0.1% benzocaine) for at least 20 min. Tissue fixation
557 and further procedures are described specifically for each case.

558

559 **Paraffin sectioning and Masson's trichrome staining**

560 Limbs were isolated and fixed with MEMFa 1x (MOPS 0.1M pH 7.4 / EGTA 2 mM /
561 MgSO₄·7H₂O 1 mM / 3.7% formaldehyde) overnight at 4°C. Samples were washed with PBS and
562 dehydrated with serial EtOH washes (25, 50, 70 and x3 100%). Samples were then incubated three times
563 with Roti®Histol (#6640, Carl Roth, Karlsruhe, Germany) at RT and four times with paraffin (Roti®-
564 Plast, #6642, Carl Roth) at 65°C in glass containers. After last incubation, samples were embedded in
565 paraffin using plastic containers and stored at RT. Longitudinal sections of 6 µM thickness were
566 obtained.

567 Masson's trichrome staining on paraffin sections was performed following the producer's
568 recommendations (Procedure No. HT15, Sigma-Aldrich). Imaging was performed in a Zeiss Axio
569 Observer.Z1 inverted microscope (objective: Plan-apochromat 20x/0.8).

570

571 **Cryosectioning**

572 Limbs fixed with MEMFa were washed with PBS and decalcified with EDTA 0.5 M at 4°C for
573 48 hours. Next, limbs were washed with PBS and incubated overnight with sucrose 30% at 4°C. Samples
574 were embedded in O.C.T. compound (#4583, Tissue-Tek, Umkirch, Germany) using plastic molds and
575 frozen with dry ice for 1 hour prior to storage at -20°C. Longitudinal sections of 12 µm thickness were
576 cut and mounted on superfrost slides. Slides were kept at -20°C until processed.

577 **TRAP enzymatic staining**

578 Tartrate-resistant acid phosphatase (TRAP) enzymatic staining was performed in cryosections.
579 Slides were dried for 1 hour prior to wash them with PBS + 0.1% Tween-20 for 10 minutes. Next, slides
580 were permeabilised with PBS + 0.3% Tx-100 for 1 hour. After permeabilization, slides were equilibrated
581 by three washes with TRAP buffer (NaAcetate 0.1M / acetic acid 0.1M / NaTartrate 50 mM / pH 5.2)
582 for 10 minutes at 37°C in water bath. Slides were stained with color reaction buffer (TRAP buffer /
583 Naphthol AS-MX phosphate 1.5 mM / Fast Red Violet LB Salt 0.5 mM) for 1 hour at 37°C in water
584 bath. After staining, slides were washed three times with PBS for 10 minutes and mounted with
585 Entellan™ (#1.07960, Sigma-Aldrich). Images were taken in a Zeiss Axio Observer.Z1 inverted
586 microscope.

587

588 **Immunofluorescence**

589 For immunofluorescence (IF), cryosections were used. Slides were dried at RT for at least 1
590 hour. Sections were washed three times with PBS + 0.3% Tx-100 prior to blocking with PBS + 0.3%
591 Tx-100 + 10% normal horse serum (NHS, #S-2000-20, Vector Labs, Burlingame, CA, USA) for 1 hour.
592 Primary anti-CTSK (#ab19027, Abcam, Cambridge, UK) antibody incubation (1:20) was done in
593 blocking solution for 1 hour at RT and then overnight at 4°C. Sections were then washed three times
594 with PBS + 0.3% Tx-100 and incubated with Goat anti-Rabbit, Alexa Fluor 647 antibody (1:200, #A-
595 21245, Invitrogen) and Hoechst 33342 1:1000 for 2 hours. Finally, sections were washed three times
596 with PBS + 0.3% Tx-100 and mounted using Mowiol mounting medium (#0713 Carl Roth). Imaging
597 was performed on a Zeiss Axio Observer.Z1 inverted microscope with an ApoTome1 system
598 (objectives: Plan-apochromat 10x/0.45 or Plan-apochromat 20x/0.8).

599

600 **RNA probes for *in situ* hybridization**

601 *Ctsk*, *Kazald1* and *Krt17* probes were created by TA cloning. Probe amplification was done
602 using primers previously published (Table II). Ligation was done into a pGEM®-T easy vector system
603 I (#A1360, Promega, Madison, WI, USA). To confirm successful cloning, vectors were purified and
604 sequenced using the Mix2Seq Kit (Eurofins Genomics, Ebersberg, Germany).

605

606 Table II: Primers for ISH probes cloning.

| Gene | Forward | Reverse | Reference |
|----------------|------------------------------|--------------------------------|-----------------------|
| <i>Ctsk</i> | GTGCAGAACCG ACCCGATG | CAGCTGGACT CGGAGTGATGC | (Bryant et al., 2017) |
| <i>Kazald1</i> | CTCGTGACATC CTGAGCCTGGAAG | GAAAATGGATAA GGTGGTGGGGAGGG | (Bryant et al., 2017) |
| <i>Krt17</i> | CCTCTTGGAC GTGAAGACC | CCAGAGAAGATGA GCATACATCGG | (Leigh et al., 2018) |

607
608 For synthesizing the ISH probes, *in vitro* transcription was carried out using a T7 polymerase
609 (#RPOLT7-RO, Roche, Mannheim, Germany) or a SP6 polymerase (#RPOLSP6-RO, Roche) following
610 provider's instructions. Prior to transcription, 5 µg of plasmid were linearized using the restriction
611 enzyme SpeI-HF® (#R3133S, New England BioLabs) for *Ctsk* and *Krt17*, or SphI-HF® (#R3182S,
612 New England BioLabs) for *Kazald1*. Probes were purified using the RNAeasy® Mini Kit (#74104,
613 QIAGEN, Hilden, Germany) according to provider's instructions.

614
615 ***In situ* hybridization (ISH)**

616 ISH was performed in cryosections using *Ctsk* probe following a previously published protocol
617 (Knapp et al., 2013). When the protocol was finished, slides were fixed in formaldehyde 4% overnight
618 at RT. Slides were then dehydrated with serial EtOH washes (25, 50, 70 and 100%) prior to wash with
619 Roti®Histol and mounting with Entellan™. Imaging was performed on a Zeiss Axio Observer.Z1
620 inverted microscope.

621
622 **Whole mount *in situ* hybridization (WISH)**

623 Whole mount ISH was performed using *Krt17* and *Kazald1* probes. Protocol was adapted from
624 (Woltering et al., 2009). Briefly, samples were dehydrated with serial MetOH washes (25, 50, 75% in
625 PBS + 0.1% Tween-20 and 100%). Limbs were bleached in MetOH + 6% H₂O₂ at RT and then
626 rehydrated with serial washes of MetOH. Then, limbs were washed with TBST (1x TBS, 0.1% Tween-
627 20) and treated with 20 µg/mL Proteinase K in TBST for 30 min at 37°C. After incubation, limbs were
628 washed with TBST and rinsed with trietanolamine 0.1M pH 7.5 (#90278, Sigma-Aldrich). Limbs were
629 incubated with freshly prepared 0.1M TEA + 1% acetic anhydride (#320102, Sigma-Aldrich) for 10
630 minutes and then washed again with TBST. Next, limbs were fixed with 4% PFA + 0.2% glutaraldehyde
631 (#G6257, Sigma-Aldrich) for 20 minutes and washed with TBST. TBST was removed and limbs were
632 incubated with previously warmed Pre-Hyb solution (hybridization buffer without probe) at 60 °C for 4
633 hours, prior to be transferred into pre-warmed hybridization buffer + probe (6 µL/mL) and incubated
634 overnight at 60°C. The next day, limbs were washed at 60°C with pre-warmed 5xSSC solution twice
635 for 30 minutes, with 2xSSC solution three times for 20 minutes, and with 0.2xSSC three times for 20
636 minutes. Limbs were then washed with TNE solution twice for 10 minutes at RT prior to incubation
637 with 20 µg/mL RNase A in TNE solution for 15 minutes. After incubation, limbs were washed with
638 TNE solution twice for 10 minutes, and with MAB solution three times for 5 minutes. Limbs were
639 blocked with MAB solution + 1% blocking reagent for 1.5 hours, and then incubated with MAB solution
640 + 1% blocking reagent + 1:3000 anti-digoxigenin-AP, Fab fragments for 4 hours at RT. Next, limbs
641 were washed with MAB solution three times for 5 minutes each and then overnight at RT. On day 3,
642 limbs were washed with MAB solution five times for 1 hour each and again overnight. After MAB
643 washes, limbs were washed with NTMT three times for 10 minutes at RT, and then incubated with

644 freshly made NTMT + 20 μ L/mL NBT/BCIP (#11681451001, Roche). For both *Kazald1* and *Krt17*
645 probes, 4-6 hours of incubation were enough for signal to develop. Reaction was then stopped by
646 incubating with PBS + 0.1% Tween-20 twice for 10 minutes and then fixing with 4% PFA at 4°C
647 overnight. After fixation, limbs were washed with PBS + 0.1% Tween-20 and stored in that solution at
648 RT. Imaging was performed on a Zeiss Discovery.V20 stereomicroscope.

649

650 **Alcian blue/alizarin red staining**

651 Staining was performed as recently described (Riquelme-Guzmán et al., 2021). Imaging was
652 performed on a Zeiss Discovery.V20 stereomicroscope (objective: Plan S 1.0x).

653

654 **Whole mount EdU staining**

655 Limbs from axolotls injected with EdU were fixed with MEMFa 1x overnight at 4°C and then
656 washed with PBS. For whole mount EdU staining, limbs were washed with PBS + 0.3% Tx-100 twice
657 for 2 hours and then blocked with PBS + 0.3% Tx-100 + 5% goat serum + 10% DMSO for 24 hours at
658 RT. Click-iT™ EdU cell proliferation kit, Alexa Fluor 488 (#C10337, Invitrogen) was used following
659 provider's instructions. Samples were incubated in reaction cocktail for 4 hours at RT. After incubation,
660 samples were washed with PBS + 0.3% Tx-100 four times for 15 min. Next, samples were incubated
661 with TO-PRO™-3 1:10.000 in PBS + 0.3% Tx-100 for 1 hour at RT. Finally, limbs were washed with
662 PBS four times for 15 min each at RT. Limbs were cleared by dehydration with serial washes of EtOH
663 (25, 50, 70, 100%) for 2 hours each at 4°C. Samples were then incubated overnight in 100% EtOH at
664 4°C prior to clearing with ethyl cinnamate (#112372, Sigma-Aldrich) at RT for at least 2 hours. Samples
665 were imaged the same day on a Zeiss confocal laser scanning microscope LSM 780.

666

667 **RNA purification and RT-qPCR**

668 Limbs for RNA isolation were stored in RNAlater™ (#AM7024, Invitrogen) at -20°C until all
669 samples were collected. RNA isolation was performed using the RNAeasy® Mini Kit. 50 ng of RNA
670 were used for cDNA synthesis using the PrimeScript™ RT reagent Kit (#RR037A, Takara, Göteborg,
671 Sweden) following the provider's instructions. RT-qPCR was performed using the TB Green® Premix
672 Ex Taq™ (Tli RNaseH Plus) kit (#RR420A, Takara). RT-qPCR was done using a LightCycler 480
673 system with a pre-defined protocol for SYBR Green. Results were analyzed using the $\Delta\Delta CT$ method
674 and the *Rpl4* housekeeping gene. After analysis, results were shown as relative levels compared to a
675 control. Primer pairs used are listed in Table III.

676

677 Table III: Primer pairs used for RT-qPCR.

| Gene | Forward | Reverse |
|----------------|-----------------------|----------------------|
| <i>Rpl4</i> | TGAAGAACCTGAGGGTCATGG | CTTGGCGTCTGCAGATTTTT |
| <i>Ctsk</i> | TGGCCCTTTAACAAACACCG | ACTGAGTTGCAACAGCTTCC |
| <i>Trap</i> | TCATTGCCTGGTCAAGCATC | TGGGCATAGTAGAACCGCAA |
| <i>Dcstamp</i> | TGGAAACCAAAAGTGCAGCG | CCCCTCAGTGCCATCATTGT |

678

679 **Polarization microscopy**

680 The LC-PolScope is a powerful tool to quantitatively image optically anisotropic materials
681 having a refractive index that depends on the polarization and propagation of light (birefringence), such
682 as collagen, the main component of cartilage ECM (Fox et al., 2009). An LC-PolScope (on a Ti Eclipse
683 microscope body) with a sCMOS camera (Hamamatsu Orca Flash 4.0) was used. Acquisitions were
684 done with a 20x/0.8 objective and using μ Manager software (Edelstein et al., 2014). Two images were
685 acquired: the retardance and the slow axis orientation. The retardance correlates with the amount of
686 birefringent components, while the slow axis orientation image provides information on the orientation
687 of those components, i.e. the angle in which they are aligned in the sample. Retardance and slow axis
688 orientation images were aligned using a custom-made MATLAB script such that the x-axis
689 corresponded to the proximodistal axis and the y-axis corresponded to the anteroposterior axis, with the
690 elbow on the top-right corner of the image. The angle was measured respect to the proximodistal axis.
691 Once the images were aligned, the regions of interest were cropped and segmented using the Trainable
692 Weka Segmentation plugin from Fiji (Arganda-Carreras et al., 2017). The segmentation was done to
693 obtain masks for the collagen regions and to remove the cells from the analysis. The masks were then
694 applied to the slow axis orientation images and the orientations of the collagen fibers were quantified
695 using MATLAB.

696

697 **Curating RNA-seq data**

698 Recently published axolotl RNA-seq datasets (Tsai et al., 2020, 2019) were used to evaluate
699 osteoclasts-related transcripts levels in samples upon full skin flap surgery. For curating the datasets, R
700 Studio was used (RStudio Team, <http://www.rstudio.com/>). In order to find osteoclasts-related
701 transcripts identifiers, the human, mouse and xenopus protein orthologous for each transcript were used.
702 With the protein sequences, a protein blast was performed using the predicted proteins from Bryant et
703 al. *de novo* axolotl transcriptome (supplementary data Bryant et al., 2017). The best three matches for
704 each protein were used for browsing in Tsai's transcriptome. The datasets from both of Tsai et al. works
705 were combined and filtered in order to have only the results from 0 dpa, 5 dpa and 5 dpa in FSF surgery.
706 In addition, the 2N, 4N and EP fractions at 5 dpa were also filtered from the combined datasets. For
707 organizing, filtering and calculating z-scores in the datasets, the tidyverse package (Wickham et al.,

708 2019) and plyr package (Wickham, 2011) were used. For creating the heatmaps, the ggplot2 package
709 was used (Wickham, 2016).

710 To find possible candidates involved in osteoclast recruitment and differentiation, a search in
711 the available literature was done for each differentially down-regulated transcript in FSF samples (385
712 transcripts, Tsai et al., 2020 supplementary data). Transcripts which have been connected to osteoclast
713 function or belong to a protein family shown to play a role in osteoclast recruitment and differentiation,
714 were filtered and heatmaps were created for better visualization of the levels during regeneration and in
715 the different fractions (Fig 18C, D).

716

717 **µCT scan**

718 Scans were performed as recently described (Riquelme-Guzmán et al., 2021). Threshold was
719 set to 220 mg HA/cm³.

720

721 **Statistical analysis**

722 Statistical analyses were performed using the software Prism9 (GraphPad Software, LLC, San
723 Diego, CA, USA) for macOS. Statistical tests performed are described in each figure. P-values < 0.05
724 were considered statistically significant.

725

726 **Image processing and figure design**

727 All images were processed using Fiji (Schindelin et al., 2012). Processing involved selecting
728 regions of interest, merging or splitting channels and improving brightness levels for proper presentation
729 in figures. Maximum intensity projections were done in some confocal images and it is stated in the
730 respective figure's descriptions. Stitching of tiles was done directly in the acquisition software Zen
731 (Zeiss Microscopy, Jena, Germany). Figures were created using Affinity Designer (Serif Europe, West
732 Bridgford, UK).

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734

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740

741 **COMPETING INTERESTS**

742

743 No conflicts of interest, financial or otherwise, are declared by the authors.

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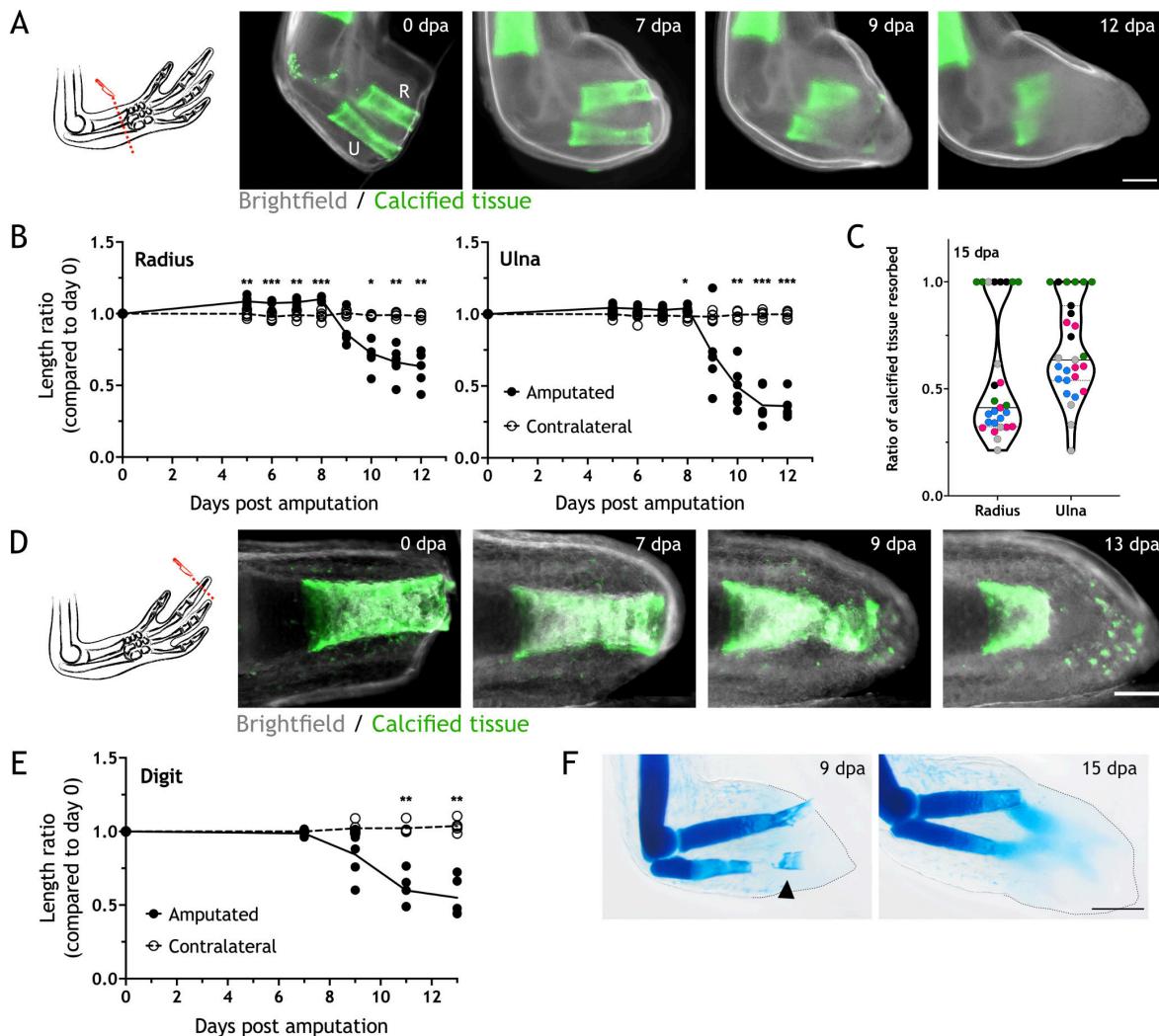
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968

969 **Figure 1: Skeletal elements are resorbed upon amputation.**

970 (A) Time course of resorption during zeugopod regeneration. Calcein-stained axolotls were amputated
 971 at the distal end of the calcified tissue (n = 6, five independent experiments). R = radius. U = ulna Scale
 972 bar: 500 μ m.

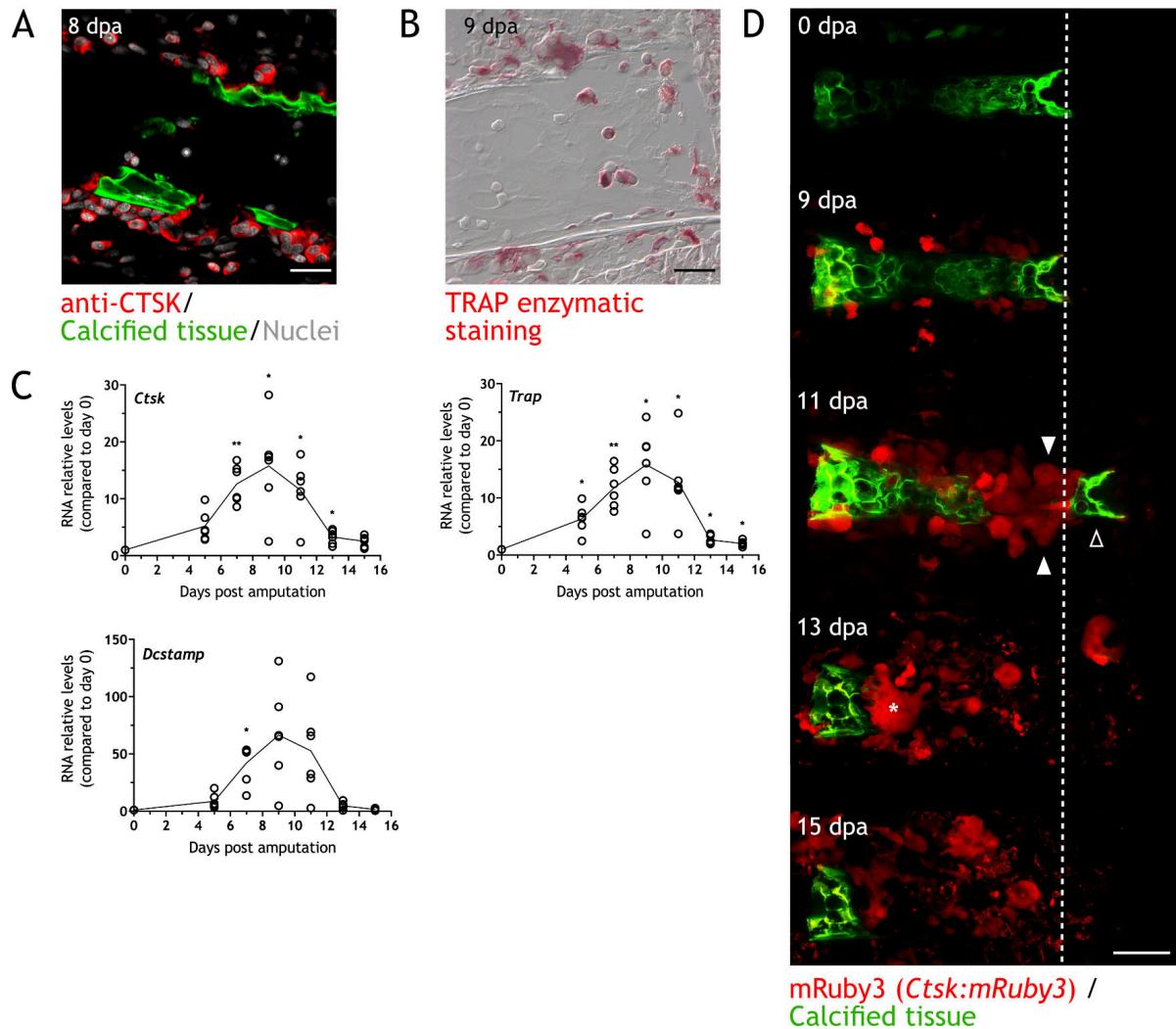
973 (B) Quantification of resorption rate in radius and ulna. Length ratio was calculated using the length at
 974 0 dpa as a reference. Each dot represents an animal (n = 6. *** p < 0.001, ** p < 0.01, * p < 0.05,
 975 Bonferroni's multiple comparisons test, amputated versus contralateral).

976 (C) Quantification of resorption percentage in calcified radius and ulna among animals in different
 977 assays. Each assay is represented by a color (n = 27, five independent experiments).

978 (D) Time course of resorption during digit regeneration. Calcein-stained axolotls were amputated at the
 979 distal end of the calcified tissue (n = 5). Scale bar: 200 μ m.

980 (E) Quantification of calcified digit resorption. Length ratio was calculated using the length at 0 dpa as
 981 a reference. Each dot represents an animal (n = 5. ** p < 0.01, Bonferroni's multiple comparisons test,
 982 amputated versus contralateral).

983 (F) Alcian blue staining of limbs at different dpa (n = 2). Arrowhead: broken piece of ulna. Dashed line:
 984 outline of distal limb. Scale bar: 500 μ m.



985

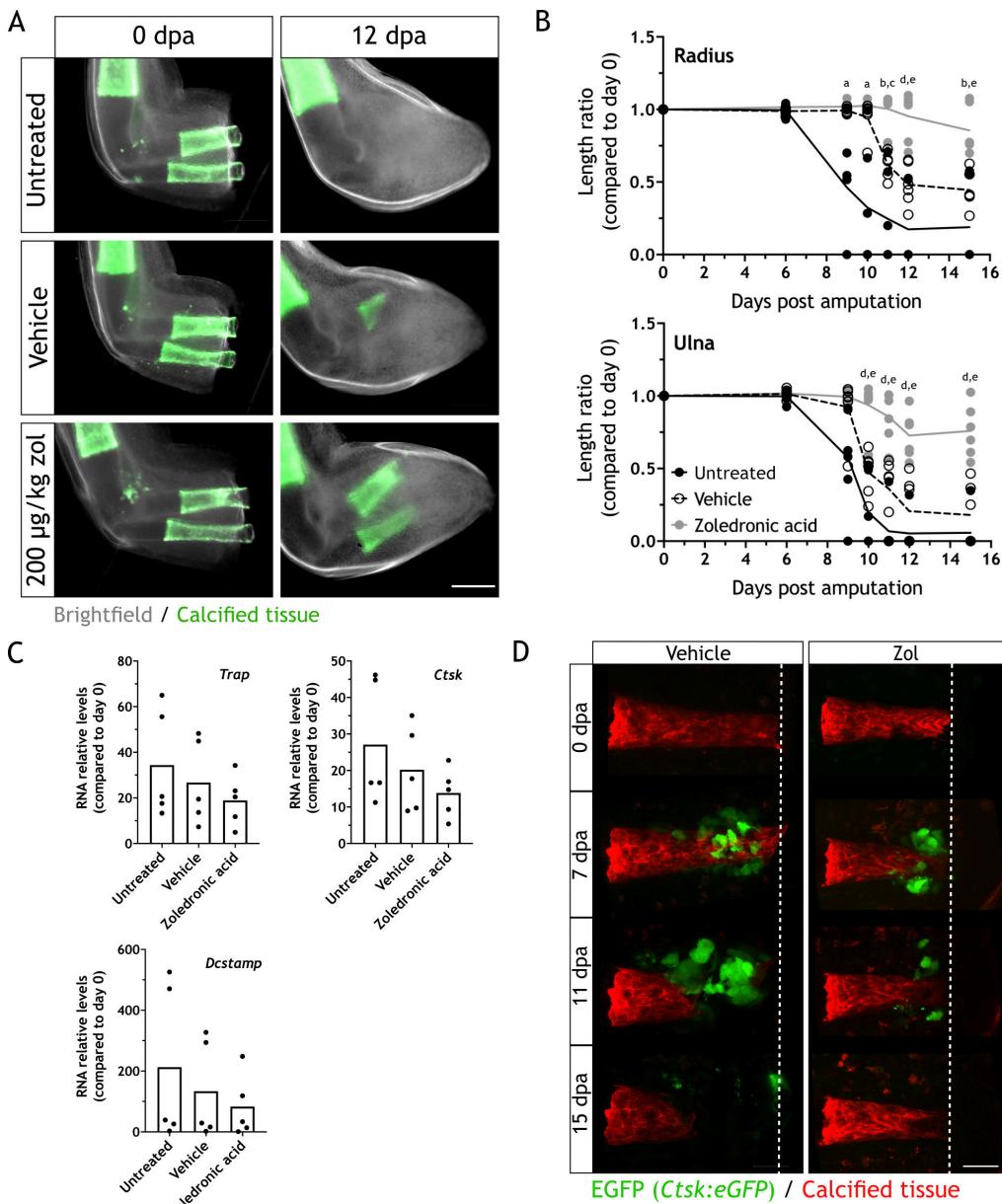
986 **Figure 2: Osteoclasts are identified during skeletal resorption.**

987 (A) Apotome image of IF for anti-CTSK (red) in zeugopod section at 8 dpa (n = 2). Calcein was used
988 for calcified cartilage labelling (green) and Hoechst for nuclear staining (white). Scale bar: 50 μ m.

989 (B) TRAP enzymatic staining in zeugopod section at 9 dpa (n = 2). Scale bar: 50 μ m.

990 (C) RT-qPCR for *Trap*, *Ctsk* and *Dcstamp* at different dpa upon zeugopodial amputation. Solid line
991 represents mean, each dot is an animal (n = 6. ** p < 0.01, * p < 0.05, Bonferroni's multiple comparisons
992 test, each time point versus 0 dpa).

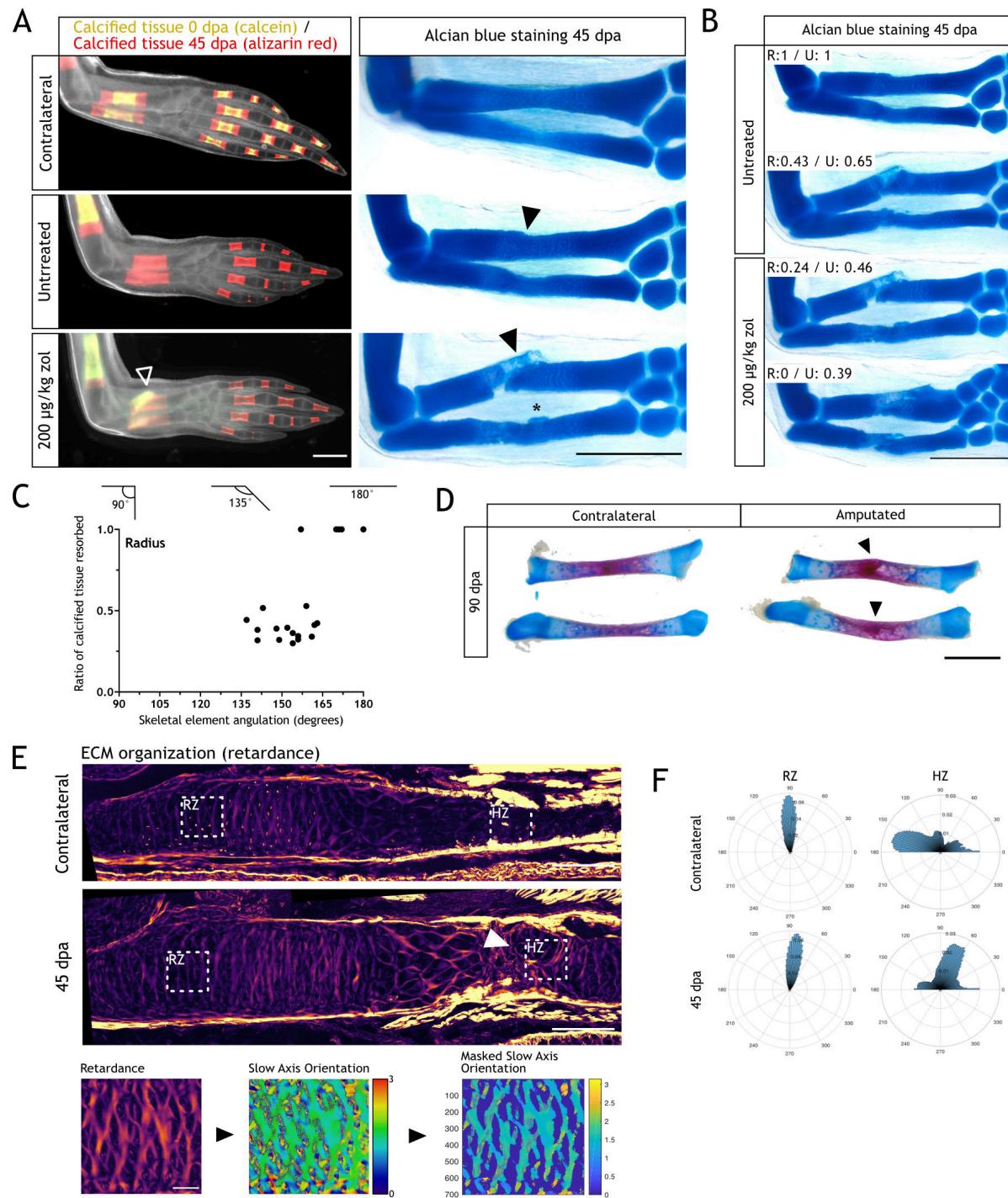
993 (D) *In vivo* confocal imaging of *Ctsk:mRuby3* (red) upon digit amputation (n = 3, two independent
994 experiments). Calcein was used for calcified cartilage labelling (green). Image represent a maximum
995 intensity projection of 10 images (3 μ m interval). White arrowhead: mRuby3⁺ cells (osteoclasts). Black
996 arrowhead: break in the skeletal tissue. Dashed line: amputation plane. Asterisk: Multinucleated
997 osteoclast. Scale bar: 100 μ m.



998

999 **Figure 3: Zoledronic acid treatment inhibits osteoclast-mediated skeletal resorption.**

- 1000 (A) Time course of resorption during zeugopod regeneration upon zoledronic acid treatment (zol) (n =
 1001 6, three independent experiments). Calcein-stained axolotls were amputated at the distal end of the
 1002 calcified tissue. Scale bar: 500 µm.
- 1003 (B) Quantification of resorption rate in radius and ulna upon zol treatment. Length ratio was calculated
 1004 using the length at 0 dpa as a reference. Each dot represents an animal (n = 6. a: p < 0.05 uninjected vs.
 1005 zol, b: p < 0.01 uninjected vs. zol, c: p < 0.001 vehicle vs. zol, d: p < 0.001 uninjected vs. zol, e: p <
 1006 0.01 vehicle vs. zol, Tukey's multiple comparisons test).
- 1007 (C) RT-qPCR for *Trap*, *Ctsk* and *Dcstamp* at 9 dpa upon zol treatment. Each dot represents an animal
 1008 (n = 5, Tukey's multiple comparisons test).
- 1009 (D) *In vivo* confocal imaging of *Ctsk:eGFP* (green) upon digit amputation (n = 4, two independent
 1010 experiments). Alizarin red was used for calcified cartilage labelling (red). Image represent a maximum
 1011 intensity projection of 15 images (3 µm interval). Scale bar: 50 µm.



1012

1013 **Figure 4: Resorption inhibition does not halt regeneration but results in an integration failure of**
1014 **the newly formed skeleton.**

1015 (A) *In vivo* calcein / alizarin red staining (left panel) and alcian blue staining (right panel) in zol treated
1016 limbs at 45 dpa (n = 6). Arrowheads: integration failure in skeletal elements. Asterisk: heterotopic
1017 cartilage formation in ulna. Scale bar: 1 mm.

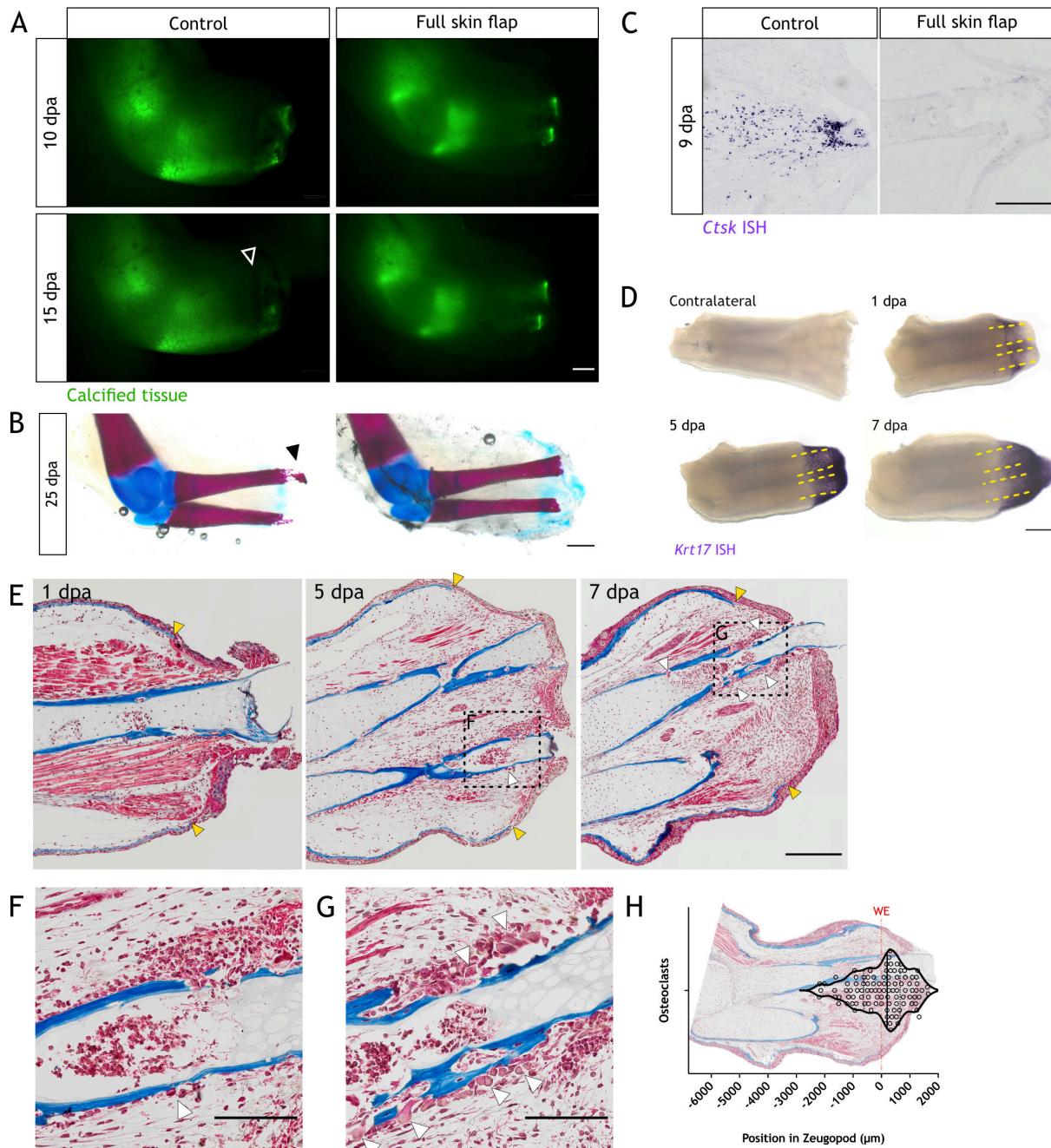
1018 (B) Alcian blue staining in zol treated limbs at 45 dpa (n = 6). Resorption rate for radius and ulna is
1019 specified for each case. Scale bar: 1 mm.

1020 (C) Quantification of angulation at the stump-regenerated interphase in untreated radii at 45 dpa.
1021 Angulation schematic is shown on top of the graph (n = 22, 4 independent experiments).

1022 (D) Alcian blue/alizarin red staining of zeugopodial elements at 90 dpa. Arrowhead: stump-regenerated
1023 interphase. Scale bar: 2 mm.

1024 (E) Upper panel: retardance image from unamputated and 40 dpa ulna. RZ (resting zone) and HZ
1025 (hypertrophic zone) squares represent the quantification areas (n = 7 for unamputated, n = 9 for
1026 amputated). Arrowhead: disorganized interphase. Scale bar: 200 μ m. Lower panel: quantification flow
1027 chart. The mask was created using the retardance image to quantify only ECM components, and applied
1028 to the slow axis orientation image to determine the orientation of the ECM components at each pixel. In
1029 the masked orientational field, the cellular regions are shown in dark blue for visualization purposes but
1030 their orientational values were excluded from the analysis in F. Scale bar: 50 μ m.

1031 (F) Histograms showing the orientation of the ECM components at each pixel in RZ or HZ for the
1032 unamputated or 40 dpa ulna (n = 7 for unamputated, n = 9 for amputated). Angles are shown in degrees.



1033

1034 **Figure 5: The wound epithelium is involved in resorption induction.**

1035 (A) Time course of resorption during zeugopod regeneration upon full skin flap surgery (n = 9). Calcein-
 1036 stained axolotls were amputated at the distal end of the calcified tissue. Arrowheads: resorption in
 1037 control cases. Scale bar: 1 mm.

1038 (B) Alcian blue/alizarin red staining of limbs at 25 dpa after full skin flap surgery (n = 9). Arrowhead:
 1039 resorption of distal radius. Scale bar: 1 mm.

1040 (C) ISH for *Ctsk* in limb sections at 9 dpa after full skin flap surgery (n = 3 for control, n = 4 for FSF).
 1041 Scale bar: 500 μ m.

1042 (D) WISH for *Krt17* in limbs upon zeugopod amputation at different dpa (n = 3). Dashed lines: skeletal
 1043 elements position. Scale bar: 500 μ m.

- 1044 (E) Masson's trichrome staining from limb sections upon zeugopod amputation at different dpa (n = 3).
1045 Yellow arrowheads: beginning of wound epithelium. White arrowheads: osteoclasts.
1046 (F) Inset from (E) 5 dpa. Scale bar: 200 μ m.
1047 (G) Inset from (E) 7dpa. White arrowheads: osteoclasts. Scale bar: 200 μ m.
1048 (H) Quantification of position of osteoclasts in zeugopod at 7 dpa. Each dot represents an osteoclast.
1049 Position of WE is shown with a red line. Image of a quantified section shows the position of osteoclasts
1050 in the sample (n = 101, 3 independent experiments).

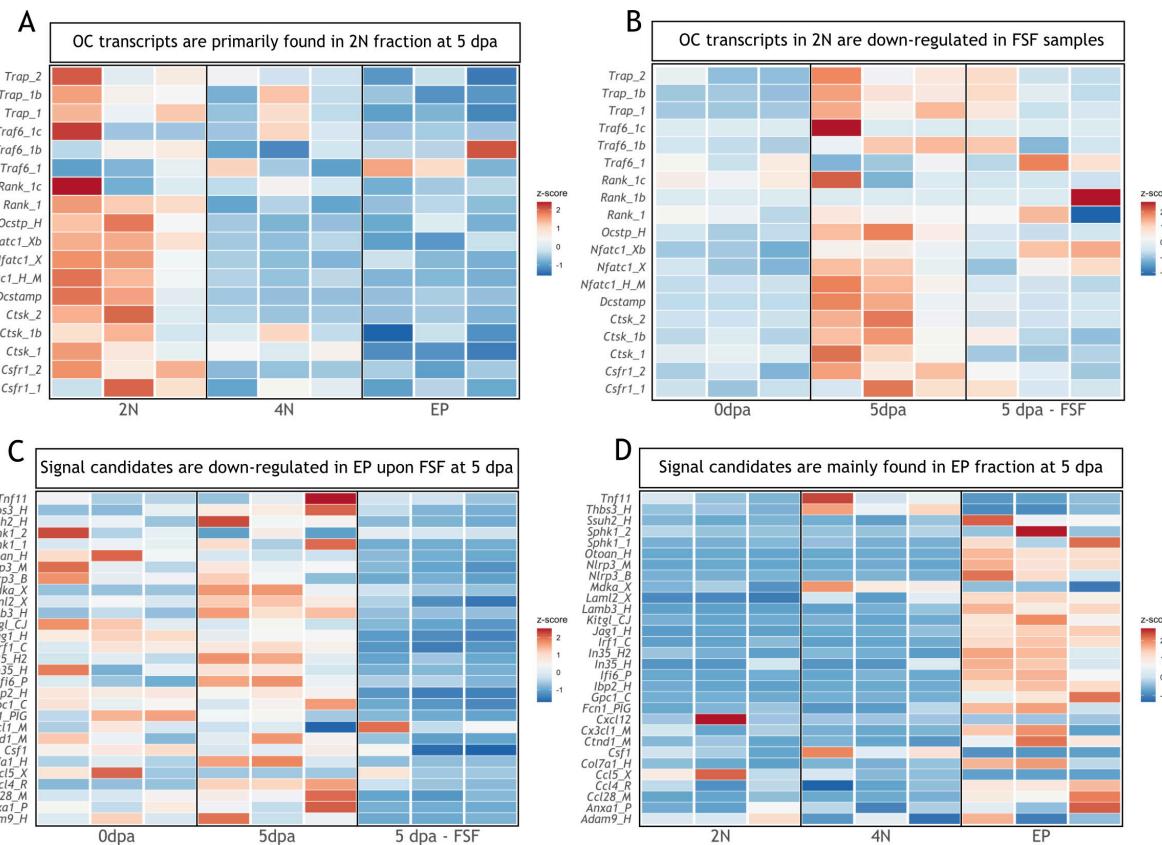


Figure 6: Transcripts associated with osteoclastogenesis are downregulated in FSF samples at 5 dpa.

(A) Heatmap of transcripts associated with osteoclast function in three different fractions at 5 dpa (n = 3). 2N: mature cells, 4N: dividing cells, EP: epithelial cells.

(B) Heatmap of transcripts associated with osteoclast function in 2N fraction at different time points (n = 3). FSF sample correspond at 5 dpa.

(C) Heatmap of differentially down-regulated transcripts in EP fraction after FSF surgery at 5 dpa associated with osteoclast recruitment and/or differentiation (n = 3).

(D) Heatmap of differentially down-regulated transcripts after FSF surgery in three different fractions associated with osteoclast recruitment and/or differentiation (n = 3).

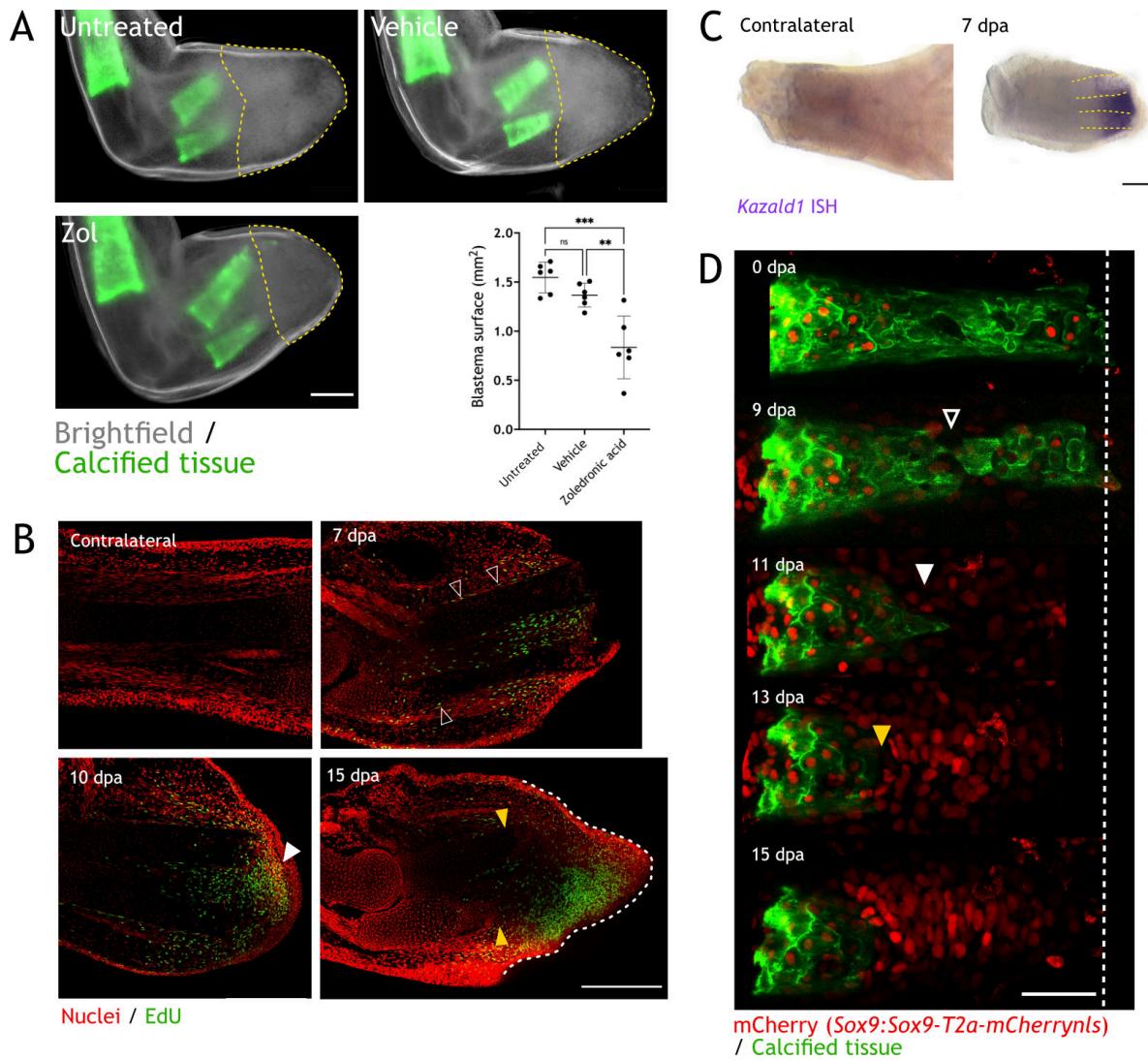
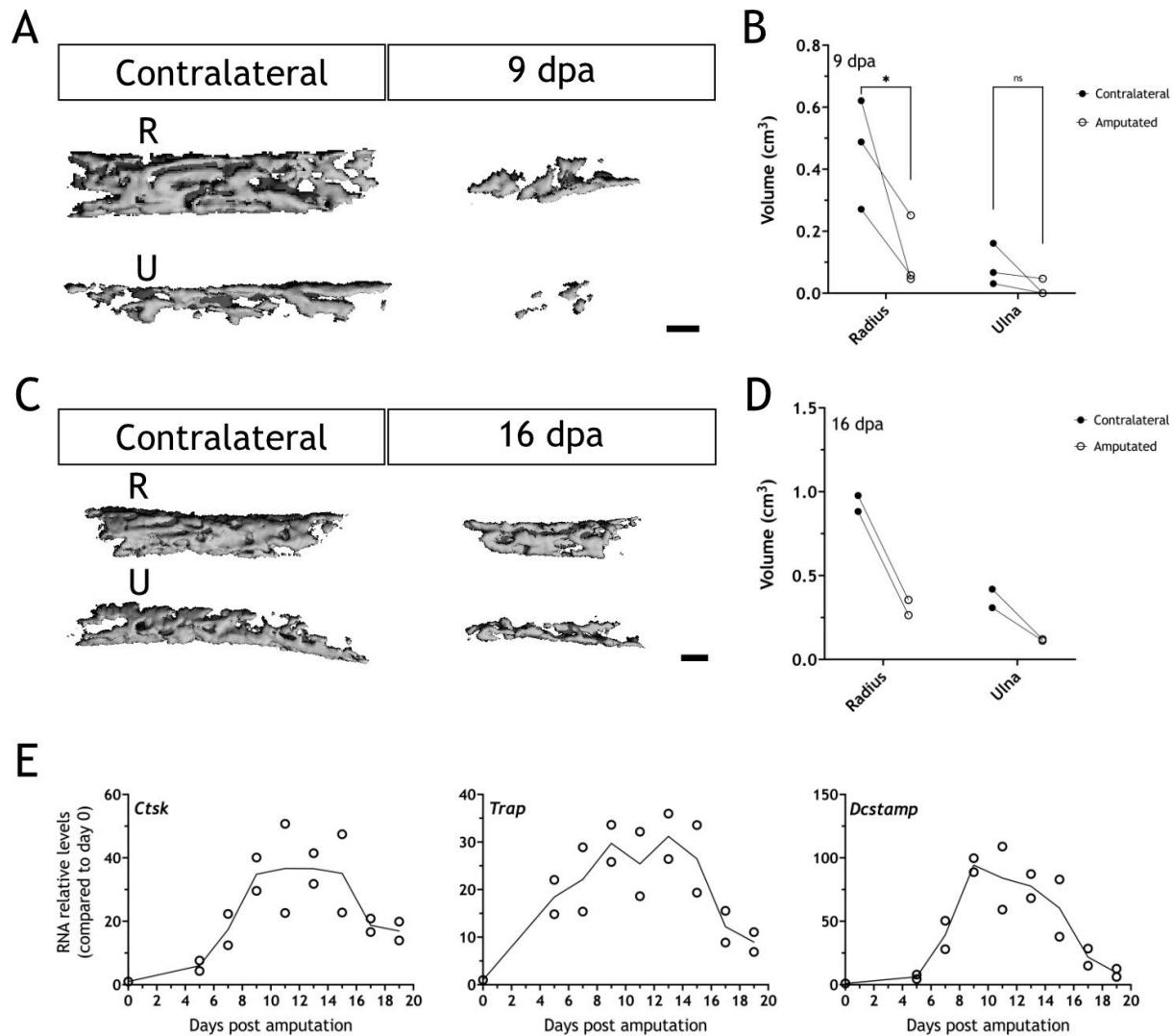


Figure 7: Skeletal resorption and blastema formation are spatially and temporally correlated.

1064 (A) Quantification of blastema size in zol treated limbs at 15 dpa. Dashed lines: blastema. Each dot
1065 represents an animal (n = 6, *** p < 0.001, ** p < 0.01, Tukey's multiple comparisons test).
1066 (B) Whole mount EdU staining (green) in limbs upon zeugopod amputation at different dpa (n = 6). TO-
1067 PRO-3 was used for nuclear staining (red). Black arrowheads: dividing periskeletal cells. White
1068 arrowhead: blastema. Yellow arrowheads: distal end of skeletal element. Dashed line: blastema. Scale
1069 bar: 500 μ m.
1070 (C) WISH for *Kazald1* in limbs upon zeugopod amputation at 7 dpa (n = 2). Yellow dashed lines:
1071 skeletal elements position. Scale bar: 500 μ m.
1072 (D) Time course of resorption during digit regeneration in *Sox9-mCherry* (red) (n = 6). Calcein-stained
1073 (green) axolotls were amputated at the distal end of the calcified tissue. Black arrowhead: calcified tissue
1074 break. White arrowhead: condensation of mCherry⁺ cells. Yellow arrowhead: resorption. Scale bar: 100
1075 μ m.



1076

Figure S1: Bones are resorbed upon amputation in 16 cm ST axolotls

1077 (A) 3D reconstructions from μ CT scans for radius (R) and ulna (U) in the contralateral and a 9 dpa limb
1078 upon amputation at the distal end of the calcified tissue ($n = 3$). Scale bar: 200 μ m.

1079 (B) Quantification of bones volume (cm^3) for samples in (A). Each dot represents an animal ($n = 3$. * p
1080 < 0.05 , Bonferroni's multiple comparisons test, contralateral versus amputated).

1081 (C) 3D reconstructions from μ CT scans for radius and ulna in the contralateral and a 16 dpa limb upon
1082 amputation at the distal end of the calcified tissue ($n = 2$). Scale bar: 200 μ m.

1083 (D) Quantification of bones volume (cm^3) for samples in (C). Each dot represents an animal ($n = 2$).

1084 (E) RT-qPCR for *Ctsk*, *Trap* and *Dcstamp* at different dpa upon zeugopodial amputation. Solid line
1085 represents mean, each dot is an animal ($n = 2$).