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1 Curcumin-mediated NRF2 induction limits inflammatory damage in preclinical models of cystic fibrosis

2
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18
19 **Author Contributions:** AB conceived the project, designed experiments, analysed data and wrote the manuscript with
20 input from SAL-I, MF, LY, RAF, SAR, J-LH, CL and CC. LY performed structural analyses. SAL-I and CC were responsible for
21 the *ex vivo* testing in organoid models. MF and CL performed SHG imaging. SC, CB and AB performed zebrafish
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35 **Abstract**

36 Overactive inflammation is directly correlated with airway damage and early death in individuals with cystic
37 fibrosis (CF), a genetic disorder caused by mutation in the *CFTR* gene. Reducing the impact of inflammatory
38 damage is therefore a major concern in CF. Several studies indicate that a decrease in the nuclear factor
39 erythroid 2-related factor-2 (NRF2) signaling in people with CF may hamper their ability to alleviate oxidative
40 stress and inflammation, although the role of NRF2 in CF inflammatory damage has not been determined.
41 Therefore, we examined whether the phytochemical curcumin, an activator of NRF2, might provide a
42 beneficial effect in the context of CF.

43 Herein, combining *Cftr*-depleted zebrafish larvae as innovative biomedical model with CF patient-derived
44 airway organoids (AOs), we sought to understand how NRF2 dysfunction leads to abnormal inflammatory
45 status and impaired tissue remodeling, and determine the effects of curcumin in reducing inflammation and
46 tissue damage in CF.

47 We demonstrate that NRF2 is instrumental in efficiently regulating inflammatory and repair processes *in*
48 *vivo*, thereby preventing acute neutrophilic inflammation and tissue damage. Importantly, curcumin
49 treatment restores NRF2 activity in both CF zebrafish and AOs. Curcumin reduces neutrophilic inflammation
50 in CF context, by rebalancing the production of epithelial ROS and pro-inflammatory cytokines. Furthermore,
51 curcumin alleviates CF-associated tissue remodeling and allows tissue repair to occur. Our findings
52 demonstrate that curcumin reduces inflammatory damage by restoring normal NRF2 activity, since
53 disruption of Nrf2 pathway abrogated the effect of treatment in CF zebrafish.

54 This work highlights the protective role of NRF2 in limiting inflammation and injury, and show that
55 therapeutic strategies to normalize NRF2 activity using curcumin might simultaneously reduce inflammation
56 and enhance tissue repair, and thus prevent infectious and inflammatory lung damage in CF.

57

58 **Keywords:** Cystic fibrosis; Curcumin; NRF2; Oxidative response; Neutrophilic inflammation; Tissue repair.

59

60

61 **Introduction**

62 Cystic fibrosis (CF), resulting from mutations in the gene encoding the CF transmembrane conductance
63 regulator (CFTR) channel, is one of the commonest fatal genetic disorders in the world (1, 2). Pulmonary
64 pathology is the prime cause of mortality and morbidity among individuals with CF and is therefore the main
65 focus of therapeutic interventions.

66 In lungs, absence or functional failure of CFTR results in an abnormal airway surface environment
67 predisposing the patients to airway obstruction, infections, inflammation and tissue remodeling (3).
68 Inflammation is a natural response of host immunity to infection or injury. However, in CF, airway
69 inflammation is both excessive and ineffective at clearing infectious burdens, causing progressive and
70 irreversible lung injury, ultimately leading to pulmonary function impairment and premature death (4).

71 The characteristic feature of airway inflammation in CF is an early, disproportionate and sustained
72 neutrophil-dominated phenotype (5). Whether inherent to the CFTR defect or in response to infections, a
73 plethora of cellular and molecular mechanisms have been proposed to explain the onset of neutrophilic
74 inflammation in CF, including the pathogenic role of epithelial oxidative response (6–8) and excessive
75 production of pro-inflammatory cytokines. Interestingly, critical regulators of redox balance, such as the
76 nuclear factor erythroid 2-related factor-2 (NRF2)-KEAP1 pathway, appear to be disrupted in several *in vitro*
77 and *ex vivo* models of CF (9–12). NRF2 is a transcription factor playing a pivotal role in the lungs to mitigate
78 oxidative and inflammatory response through the regulation of genes involved in oxidative stress,
79 antioxidant defense and pro-inflammatory processes *via* the antioxidant response elements (AREs) signaling
80 pathway (13). Although CF epithelia exhibit reduced NRF2 activity and have been associated with increased
81 production of reactive oxygen species (ROS) and pro-inflammatory cytokines (9), it remains unclear whether
82 the excessive neutrophilic inflammation in CF is related to defective NRF2 function.

83 In CF care, the use of conventional anti-inflammatory therapies, such as corticosteroids and ibuprofen,
84 can sometimes be effective in limiting inflammatory damage, but their adverse effects have discouraged
85 their long-term use and new treatment options are desired (14). Considering the protective role of NRF2,
86 therapeutic strategies designed to activate NRF2 may have clinical benefit in CF by reducing oxidative stress,
87 inflammation and subsequent cell damage in the lungs. Curcumin, widely known for its anti-inflammatory
88 and antioxidant properties, is a potent NRF2 activator (15). However, the biological significance of curcumin
89 treatment in CF-related inflammatory damage is not known.

90 Using *cftr*-depleted zebrafish (7, 16) as an innovative and relevant vertebrate model of CF, combined with
91 CF patient-derived airway organoids (AOs) (10), we demonstrated that curcumin, by normalizing NRF2
92 activity, can alleviate neutrophilic inflammation in CF context by reducing oxidative response and the levels
93 of pro-inflammatory cytokines. In addition, curcumin directly mitigates CF-associated tissue damage and
94 allows tissue repair to occur. These findings serve as supportive evidence for future evaluation of curcumin
95 in people with CF.

96

97 **Results**

98

99 **Zebrafish and human NRF2 proteins are structurally conserved**

100 Two putative co-orthologs of human NRF2 (hNRF2) have been described in zebrafish, Nrf2a and Nrf2b (17,
101 18). Sequence comparison revealed that Nrf2a resembles hNRF2 more closely than Nrf2b as it shares 46%
102 sequence identity with the human protein and displays a similar domain organization, with seven Neh
103 domains and highly conserved consensus motifs, in particular in the Neh1 (ARE-binding) and Neh2 (KEAP1-
104 binding) domains (**Figures 1A; S1**). In contrast, Nrf2b diverges a bit more from the human protein, with only
105 32% sequence identity, long deletion regions and only six conserved Neh domains, the Neh4 domain being
106 absent (**Figures S1; S2A**). Despite some degree of conservation, sequence identity remains medium between
107 hNRF2 and the zebrafish Nrf2a and Nrf2b, which questions the true functional orthology between these
108 proteins.

109 To further address their resemblance, we performed structural analysis of the three proteins (hNRF2,
110 Nrf2a and Nrf2b). As no experimental structure has been described for zebrafish Nrf2a yet, we used
111 AlphaFold to predict its protein structure (19). As shown in **Figure 1B**, Nrf2a is proposed to adopt a largely
112 unstructured 3D-architecture, alternating long coiled-coil regions with shorter, well-structured domains that
113 correspond to the functional core of the Neh domains and are predicted with high confidence. In contrast,
114 the low confidence of the prediction for coiled-coil regions suggests that they may fold differently when
115 interacting with binding partners. Comparison of the AlphaFoldF model of Nrf2a with that of hNRF2 (**Figure**
116 **1C**) reveals a high structural similarity. In particular, the predicted secondary structure elements are placed
117 at the same position in Nrf2a and hNRF2. Furthermore, the Neh1 and Neh2 domains of Nrf2a align well with
118 the corresponding domains of hNRF2 from both the predicted AlphaFold model and available experimental
119 structures of isolated domains (**Figures 1D-E**) (20, 21). A similar structural analysis was performed on Nrf2b
120 and predicts a closely related structural organization although somewhat more divergent than hNRF2 (**Figures**
121 **S2B-E**).

122 Taken together, these data suggest that zebrafish Nrf2 proteins, and in particular Nrf2a, are structural
123 homologs of hNRF2, reinforcing the idea that they ensure similar biological functions, and thus validating the
124 use of zebrafish model to study the function of NRF2 *in vivo*.

125

126 **CF-mediated NRF2 deficiency promotes overactive inflammation *in vivo***

127 To gain further insight into the impact of a dysfunctional CFTR on NRF2 activity *in vivo*, the expression of
128 *nrf2a* and *nrf2b* in normal (control) and *cftr*-depleted (*cftr* morphant (MO)) zebrafish larvae was first
129 evaluated following injury-induced inflammation (**Figure 2A**, (7)). While injury consistently triggered *nrf2a*
130 response in control larvae, comparative RT-qPCR analyses revealed a significant downregulation of *nrf2a*
131 level expression in CF animals (**Figure 2B**). Conversely, we found that *cftr* deficiency resulted in increased
132 expression of *nrf2b* compared to the normal condition (**Figure 2B**).

133 Next, to determine how disrupted NRF2 activity might contribute to the hyper-inflammatory state in CF,
134 expression of *nrf2a* and/or *nrf2b* were knocked-down in zebrafish using established morpholinos (18). As an
135 exuberant influx of neutrophils is the hallmark of CF-related inflammation, we first investigated the
136 consequence of Nrf2 inhibition on neutrophil response following injury by exploiting the
137 *TgBAC(mpx:EGFP)i114* reporter line labeling neutrophils (22). Only zebrafish injected with the *nrf2a*
138 morpholino were characterized by an increased recruitment of neutrophils to the wound compared to those
139 injected with the control one, while no significant change was observed from animals injected with the *nrf2b*
140 morpholino (**Figures 2C-D**). These results suggest that Nrf2a acts as a main regulator of neutrophilic
141 inflammation in zebrafish, whereas Nrf2b shows no obvious function. To note, neither Nrf2a nor Nrf2b
142 ablation had measurable impact on the total number of neutrophils (**Figures S3A-B**). Importantly, a similar
143 increase in the number of wound-associated neutrophils was observed in *cftr*- and *nrf2a*-depleted larvae,
144 and in the double *cftr/nrf2a*, *cftr/nrf2b* and *cftr/nrf2a+b*-depleted animals (**Figure 2E**). This is consistent with
145 the hypothesis that NRF2 signaling is already disrupted in CF and might be involved in the excessive
146 neutrophilic inflammation seen in this disease. We furthermore modulated NRF2 activity pharmacologically
147 using the NRF2 blocker ML385 or the NRF2 activator bardoxolone methyl (CDDO-Me). Corroborating our
148 finding using genetic approaches, we also observed that ML385 markedly exacerbated neutrophil response
149 in wild-type animals (**Figure 2F**). In contrast, a protective effect of CDDO-Me exposure, acting to reduce
150 neutrophilic inflammation, was demonstrated in CF larvae (**Figure 2F**). These findings suggest that NRF2
151 activation might alleviate excessive neutrophil responses in CF animals.

152 ROS generation by the dual oxidase DUOX is known to induce neutrophil chemoattraction (23). As shown
153 in **Figures S4A-B**, pharmacological inhibition of ROS production reduced neutrophil response in *nrf2a*-
154 depleted zebrafish, suggesting that exaggerated neutrophil mobilization associated with the loss of Nrf2a is
155 due to a disrupted oxidative activity. To explore this hypothesis and to investigate the role of NRF2 in
156 regulating redox balance, we next addressed whether Nrf2a ablation could affect oxidative responses in
157 zebrafish. As expected, Nrf2a deficiency caused abnormal elevation of ROS production at the wound (**Figures**
158 **S4C-D**), coinciding with increased expression of *duox* (**Figure 2G**). In contrast, *nqo1* and *hmox1*, two anti-
159 oxidative NRF2 target genes, were downregulated in animals lacking Nrf2a (**Figure 2G**). Moreover, we found
160 that loss of Nrf2a led to increased expression of pro-inflammatory genes *il8* and *il1 β* (**Figures 2G; S4E-F**), two
161 other important mediators of neutrophil chemotaxis (**Figure S4G**, (7)) known to be regulated by NRF2 (24,
162 25).

163 Collectively, these observations provide evidence that NRF2 activity is necessary to coordinate neutrophil
164 trafficking. In particular, we show that *cftr*-depleted zebrafish recapitulate the abnormal NRF2 status
165 associated with CF and support that dysfunctional NRF2 activity in CF airways contributes to neutrophilic
166 inflammation, likely by causing deleterious changes in oxidative and pro-inflammatory responses.

167

168 **CF-mediated NRF2 deficiency alters tissue repair *in vivo***

169 Our previous work revealed that *cftr*-depleted zebrafish exhibit impaired tissue repair after injury (7).
170 Considering the protective role of the NRF2 signaling pathway in repair processes (26), we next investigated
171 whether dysfunctional NRF2 activity might impede tissue repair in CF. Since zebrafish caudal fins are known
172 to undergo complete regeneration after amputation, we assessed host regenerative performance in the
173 absence of Nrf2 signaling by quantifying fin regrowth post-amputation (**Figure 3A**, (7)). Fin regrowth was
174 impaired in *nrf2a*-depleted zebrafish compared to controls, in association with a reduction in the area, length
175 and width of regenerated fins (**Figures 3B-C**), whereas the loss of Nrf2b did not affect regrowth (**Figure S5**).
176 These findings indicate that Nrf2a is the likely NRF2 paralogue that mediates tissue repair mechanisms in
177 zebrafish. Importantly, similar regenerative defects were observed in both *cftr* and *nrf2a* MO, and in the
178 double *cftr/nrf2a* MO (**Figure 3D**), suggesting that impaired tissue repair in CF zebrafish could be linked to
179 defective Nrf2a. We next reasoned that excessive neutrophilic inflammation at wound (**Figure 2**) might
180 contribute to the impaired tissue repair in *nrf2a*-depleted zebrafish. To address this question, we ablated
181 neutrophils in zebrafish using the *csf3r* morpholino (27). As shown in **Figure S6**, removal of neutrophils
182 significantly improved fin regrowth in *nrf2a* morphant animals.

183 It is known that NRF2 influences extracellular matrix (ECM) deposition and remodelling, including collagen
184 formation (28), a critical step during tissue repair. In zebrafish, collagen projections at the wound edge guide
185 epithelial growth during caudal fin regeneration and initiate tissue repair ((29, 30); **Figure 3E**). Since optimal
186 tissue repair requires a functional NRF2 activity, we sought to characterize the effect of *nrf2* ablation on the
187 formation of collagen projections during the process of regeneration. Using second harmonic generation
188 (SHG) imaging to visualize collagen fibers, we found that the formation of collagen projections was disrupted
189 in *nrf2a*-deficient larvae (**Figures 3F-I**). While the wound edge in control animals formed an uneven border
190 containing collagen associated-epithelial projections, *nrf2a*-depleted larvae displayed a smooth wound edge
191 and relative absence of such projections (**Figure 3F**). Following amputation, $\approx 90\%$ of control larvae had
192 visible projections while only $\approx 50\%$ of *nrf2a*-depleted larvae displayed projections (**Figure 3G**). In addition,
193 analyses revealed a reduced number of collagen projections in *nrf2a*-depleted animals compared to controls
194 (**Figure 3H**). Finally, the contour of the wound edge was measured in control and *nrf2a* MO, as a marker of
195 collagen fiber formation as well as a state of active tissue repair (29). The *nrf2a*-deficient larvae had a shorter
196 contour length at the wound edge as compared to control larvae (**Figure 3I**). These findings indicate that
197 Nrf2a deficiency impairs wound repair in zebrafish, at least in part through affecting the formation of collagen
198 and epithelial projections.

199 Altogether, these findings provide further evidence that functional NRF2, by regulating collagen
200 formation, is required for efficient tissue repair, and support the proposal that impaired NRF2 activity in CF
201 airways could be involved in the abnormal tissue remodeling seen in this disease (31, 32).

202

203 **Curcumin-induced Nrf2 activation reduces CF-related inflammation in zebrafish**

204 Current therapies targeting inflammation in CF have limited efficacy. Since NRF2 signaling represents an
205 important mechanism in the regulation of inflammatory and tissue repair processes, it could be considered
206 as a promising therapeutic target to prevent excessive inflammation and tissue damage in CF. We therefore
207 examined whether turmeric-derived curcumin, a NRF2 activator, might provide a beneficial effect in the
208 context of CF (**Figure 4A**).

209 Firstly, we confirmed that curcumin treatment was able to rescue disrupted *nrf2* expressions in *Cftr*-
210 depleted zebrafish (**Figures 4B; S7**), and could promote Nrf2a activation. Curcumin efficiently reduced early
211 neutrophil response to injury in CF larvae compared to DMSO-treated animals (**Figures 4C-D; S8**).
212 Interestingly, despite the anti-inflammatory properties of curcumin, neutrophilic inflammation was similar in
213 DMSO- and curcumin-treated larvae in the presence of normal *Cftr* activity (**Figures 4C; S8**). Additionally,
214 since pulmonary infections are major features in CF (33), we were also interested in determining the effect
215 of curcumin on neutrophilic inflammation in a context of infection. To do so, we used a range of CF-relevant
216 bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Mycobacterium abscessus*, added
217 into the media of injured larvae (**Figure S9A**). Microscopy observations revealed that neutrophil recruitment
218 was significantly increased in response to the bacteria-infected injuries (**Figure S9B**). Having proven that
219 curcumin does not alter bacterial growth *in vitro* (**Table 1**), we treated infected-CF zebrafish with curcumin
220 and showed that the compound also alleviated hyper-neutrophilia in a context of infection (**Figure S9C**).

221 To validate the NRF2-targeted action of curcumin in our system, we knocked-down *nrf2a*, *nrf2b* or *nrf2a+b*
222 expression in zebrafish and examined whether curcumin was still able to restore normal neutrophil responses
223 in the absence of Nrf2 signaling. As shown in **Figure S10**, although wound-associated neutrophil number was
224 significantly higher in *nrf2a*-depleted larvae than control animals when treated with curcumin, the treatment
225 partially reduced neutrophilic inflammation in the absence of Nrf2a. However, when both *nrf2a* and *nrf2b* were
226 depleted, curcumin failed to reduce neutrophil inflammation, supporting the notion that curcumin regulates
227 neutrophil response through Nrf2-dependent mechanisms. Importantly, when *nrf2a* was depleted in CF
228 animals, curcumin failed to reduce inflammation (**Figure 4E**), confirming that the presence of functional Nrf2
229 proteins, at least Nrf2a, is required for the anti-inflammatory action of curcumin to occur.

230 Excessive nuclear factor- κ B (NF- κ B) pathway activation has been described in the pathology of lung
231 inflammation in CF (34), and was confirmed in zebrafish, with higher NF κ B signal at wound in *Cftr*-depleted
232 zebrafish compared to control animals (**Figures S11A-C**). In parallel of NRF2 activation, curcumin can directly
233 inhibit NF κ B signaling pathway (35). Therefore, the NF κ B inhibitory action assigned to curcumin could also
234 be considered. However, we found that curcumin exposure was not sufficient to reduced Nfkb signal in *cftr*
235 MO (**Figure S11D**), suggesting that curcumin doesn't reduce inflammation in CF through NF κ B inhibition.

236 We next sought to understand the mechanisms by which curcumin-induced NRF2 activation reduces
237 neutrophilic inflammation in CF animals. Since exaggerated production of ROS by epithelia contributes to the
238 inflammatory pathogenesis in CF (36), we first proceeded to examine the potential benefits of curcumin in
239 reducing oxidative stress in CF larvae. Unexpectedly, rapidly after laser-induced epithelial tissue injury,

240 dynamic imaging and quantitative analyses revealed that curcumin-treated larvae exhibited a strong
241 oxidative burst at the injury site (**Figures 4F-H**). However, while oxidative responses increased over time in
242 DMSO-treated larvae, our observations indicated that curcumin gradually reduced ROS signal, suggesting
243 that treatment might reduce ROS generation through the modulation of NRF2-induced genes. As expected,
244 the anti-oxidative action of curcumin was confirmed by the downregulation of *duox* and the upregulation of
245 *nqo1* gene in CF larvae treated with curcumin (**Figures 4I-K**), consistent with Nrf2 activation. Moreover,
246 curcumin exposure ameliorated the increased Il8 and Il1 β activity caused by CF, as demonstrated by qRT-PCR
247 and/or microscopy analyses (**Figures 4L-O; S11E-G**).

248 Together these data indicate that curcumin efficiently alleviates CF-related neutrophilic inflammation, by
249 reducing oxidative and pro-inflammatory responses, *via* NRF2 activation.

250

251 **Curcumin prevents tissue damage by improving tissue remodeling and repair in CF zebrafish**

252 Pulmonary fibrosis and abnormal tissue remodeling disrupt lung function and cause death in CF.
253 Considering the role of NRF2 pathway in repair processes (**Figure 3**), we next investigated whether curcumin-
254 induced Nrf2a activation could rescue abnormal tissue remodeling and repair in CF zebrafish (7). As shown
255 in **Figures 5A-C**, curcumin exposure significantly improved tissue repair in CF injured zebrafish. Furthermore,
256 while the presence of bacteria worsened tissue damage and defective tissue repair phenotype (**Figures S12A-**
257 **B**), curcumin was also able to enhance tissue repair in CF fish in the context of infection (**Figure S12C**).

258 Because proper collagen fiber production and reorganization are essential for functional tissue repair in
259 zebrafish (30), we sought to further determine whether the deficit in caudal fin regeneration in CF zebrafish
260 was associated with a defect in collagen remodeling. Firstly, comparative analysis of the control and *Cftr*-
261 depleted zebrafish regenerated tissue revealed that collagen fibers did not project well in CF animals (**Figures**
262 **6A-D; S13A**). Furthermore, SHG imaging showed that collagen fibers were completely disrupted upon injury
263 in CF animals, as measured by the area devoid of fibers (**Figures 6E-G; S13B**). Fiber alignment analysis (**Figures**
264 **6H-L**) indicated that this change correlates with a diminished number of fibers (**Figure 6I**) and a reduction in
265 their lengths (**Figure 6J**). Importantly, a significant change in collagen fiber width was seen in *cftr* MO, with
266 thicker fibers compared to control MO (**Figure 6K**), suggesting that *Cftr* influences injury-induced fiber
267 thickening. Interestingly, this change was associated with an overexpression of the transforming growth
268 factor- β (Tgf- β) (**Figure 6L**), a key pro-fibrotic cytokine / primary driver of fibrosis in CF (37). The population
269 of fibers in CF fins also tended to be less perpendicular overall to the wound edge than their control
270 counterparts (**Figure 6M**). Overall, regenerated tissues in CF animals consistently showed unaligned fibers
271 compared to control, and were more prone to develop aberrant fiber extrusions into the intra-fiber space
272 (**Figure 6N**). Interestingly, while mesenchymal cells are essential to guide the collagen fibers organization in
273 zebrafish fins (38), we observed that disrupted fiber content and orientation coincided with an abnormal
274 mesenchymal cell distribution and shape (**Figure S13C**). Remarkably, all these aberrant phenotypes are
275 rescued by curcumin treatment (**Figures 6; S13**).

276 Together these data indicate that curcumin efficiently improves tissue repair and prevents tissue damage
277 in CF animals, by restoring remodeling of collagen and tissue.

278

279 **Curcumin reduces inflammation and epithelial damage in CF patient-derived airway organoids**

280 Finally, to further confirm the efficacy of curcumin in both reducing inflammation and tissue damage in
281 CF, while extending our results into a human system, we validated the effect of curcumin on bronchiolar
282 airway organoids (AOs)-derived from people with and without CF (10, 39). While CF-driven dysfunctional
283 NRF2 activity was confirmed by the reduction in expression of *NRF2* in CF-AOs compared to healthy-AOs (10),
284 we first showed that treatment with curcumin significantly increased *NRF2* expression in CF-AOs (**Figure 7A**).
285 We showed that curcumin treatment brought *IL1 β* and *IL8* expression back to normal level in CF-AOs (**Figure**
286 **7B**). Moreover, we evaluated the expression of genes related to the production and detoxification of ROS
287 and observed that CF-AOs treated with curcumin exhibited a mitigated oxidative response associated with
288 reduced expression of *DUOX1* and higher level expressions of *NQO1* (**Figure 7C**).

289 As previously described, CF AOs display increased cell death and thicker epithelium compared to healthy
290 ones (10), reflecting abnormal epithelial damage and remodeling. Having shown that curcumin efficiently
291 limits tissue damage in CF zebrafish, curcumin treatment was next evaluated for its effects on cell integrity
292 and mortality in AOs. Our results indicate that CF-AOs treated with curcumin displayed a significant decrease
293 in cell mortality, when assessed by propidium iodide (PI) staining (**Figures 7D-E**). Furthermore, as shown in
294 **Figures 7F-G**, treatment reduces wall thickness in CF AOs, denoting an improvement of cell integrity.

295 Altogether, these data support the potential of curcumin as a therapeutic for inflammatory lung damage
296 in CF.

297

298

299 **Discussion**

300 Over the last decade, research progress has led to spectacular advances in the management of CF, in
301 particular with CFTR correctors and potentiators correcting the basic defect of CFTR function (40). However,
302 excessive inflammation continues to be a major cause of lung destruction in CF, indicating a continued need
303 for novel strategies to prevent inflammation-related pulmonary decline.

304 Among the defective mechanisms specific to CF-related inflammatory pathogenesis, the NRF2 signaling
305 pathway, a master regulator of oxidative, inflammatory and regenerative responses, represent a promising
306 target. Indeed, reports have shown differential expression of NRF2 expression and activation in several *in*
307 *vitro* and *ex vivo* models of CF. Pharmacological activation of NRF2 results in decreased markers of oxidative
308 and pro-inflammatory responses (9–12), suggesting that rebalancing NRF2 activity might simultaneously
309 prevent inflammation and tissue damage in individuals with CF.

310 In the present study, using a combination of innovative animal and human models of CF, we sought to *i*)
311 further explore the role of NRF2 in CF-related inflammatory status and associated tissue damage, and *ii*)
312 determine the beneficial effect of curcumin, a known activator of NRF2, on inflammation and tissue repair
313 in the context of CF. Our work identifies, for a first time, a role for NRF2 in neutrophilic inflammation, tissue
314 damage and remodeling in CF. Importantly, we show that curcumin can restore normal level of inflammation
315 and tissue integrity in a context of CF, by rescuing NRF2 signaling.

316 The link between NRF2 dysfunction and CF-associated neutrophilic inflammation remained elusive,
317 although evidence suggests that impaired NRF2 activity correlates with excessive generation of ROS and pro-
318 inflammatory cytokines in CF airway. Our results reveal that loss of Nfr2 signaling leads to an exuberant
319 neutrophil response after injury in zebrafish. It coincides with an excessive production of epithelial ROS and
320 pro-inflammatory cytokines IL8 and IL1 β , all well-known key inducers of neutrophil mobilization (**Figure 2**).
321 These data provide evidence that NRF2 pathway is instrumental to efficiently orchestrate neutrophil
322 trafficking, likely by regulating oxidative and pro-inflammatory responses. Importantly, corroborating data
323 from other CF models (9–12), Cfr-depleted zebrafish model also exhibits impaired Nrf2 signaling. Our
324 findings confirm that impaired Nrf2 contribute to the neutrophilic inflammatory status in CF, since
325 pharmacological NRF2 activation can block this effect in CF animals (**Figure 2**). The plausible explanation is
326 that NRF2 dysfunction in CF leads to excessive production of ROS and pro-inflammatory cytokines by
327 epithelia in response to injury or infection (or inherent to CFTR mutations), which in turn, cause exuberant
328 neutrophilic inflammation.

329 Consistent with previous studies suggesting a role for NRF2 in tissue repair (26), we show that zebrafish
330 lacking Nrf2 signaling exhibit incomplete tail fin regrowth after amputation, associated with disrupted
331 collagen fiber formation and cell damage (**Figure 3**). Ablation of neutrophil in *nrf2a*-depleted animals
332 partially rescues tissue repair, suggesting that neutrophilic inflammation is not the only cause of defective
333 tissue repair in this context, and thus indicates that additional NRF2-mediated mechanisms, beyond its anti-
334 inflammatory/antioxidant actions, are likely to participate in tissue repair processes. Similarly, we previously

335 reported that Cfr-depleted zebrafish had abnormal tissue repair, partly due to an overactive inflammation
336 (7). This also appears to be NRF2 dependent, since pharmacological activation of NRF2 signalling improves
337 tissue repair in CF fish (**Figures 3,5-6**). These findings strongly suggest that deleterious changes in NRF2
338 activity are involved in defective tissue repair and tissue damage in CF.

339 Overall, these findings demonstrate the role of Nrf2 in CF-related inflammation and tissue damage, and
340 thus provide a rationale for targeting this pathway to prevent inflammatory damage in patients. Curcumin,
341 primary constituent in turmeric derived from *Curcuma Longa* rhizomes, is a potent activator of the NRF2
342 signaling pathway with anti-inflammatory, antioxidant and anti-infective therapeutic properties (41). We
343 proposed here to study efficiency of curcumin on inflammatory response and tissue integrity in a context of
344 CF, attempting to alleviate inflammation and tissue damage in CF.

345 We confirm that NRF2 activity is restored by curcumin treatment in both Cfr-depleted zebrafish and CF AOs
346 (**Figures 4 and 7**). NRF2 is activated by stress (42); KEAP1 allows NRF2 to escape degradation, accumulate
347 within the cell, and translocate to the nucleus, where it can bind to AREs promoting antioxidant and anti-
348 inflammatory transcription programs. Interestingly, despite the large amounts of ROS produced in CF airways,
349 NRF2 signalling fails to be activated and translocated to the nucleus and is rapidly degraded in CF (9, 11). Notably,
350 several mechanisms could be proposed to explain the stimulating action of curcumin on NRF2 in a context of CF
351 (15) : by inhibiting KEAP1, by affecting the upstream mediators of NRF2 influencing the expression of *NRF2* and
352 target genes, and/or by improving the nuclear translocation of NRF2.

353 Our results indicate that curcumin can significantly reduce CF inflammation *in vivo* and *ex vivo* by rebalancing
354 excessive generation or inefficient detoxification of ROS, as well as elevated expression of *IL8* and *IL1 β* .
355 Consequently, curcumin alleviates CF-mediated neutrophilia. While curcumin has multiple effects and modes
356 of action (41), we show that ablation of NRF2 signaling in CF zebrafish abolishes the protective effect of
357 curcumin on CF-induced inflammation. Although we cannot exclude the possibility that curcumin modulates
358 additional mechanisms to reduce inflammation, our conclusions provide evidence that curcumin prevents
359 CF-mediated inflammation *via* activating the NRF2 pathway.

360 Since curcumin is safe and well-tolerated (43), these findings could have significant therapeutic implications
361 for potently targeting inflammation in CF lung disease, and thus may serve as supplements to current
362 therapeutic strategies or be an alternative to existing anti-inflammatory approaches. These data also suggest
363 that curcumin may have beneficial effects on the extrapulmonary co-morbidities occurring in people with CF,
364 such as gastro-intestinal and colorectal cancers (44, 45) or pancreas destruction and diabetes (46, 47). While
365 CF is principally characterized by pulmonary disease, CF-related diabetes resulting from pancreatic islets
366 destruction involving inflammation is a common feature among the individuals with CF and is associated with
367 accelerated lung decline and increased mortality. CF pancreas is indeed subject to high level of ROS and *IL1 β* ,
368 both known to cause β -cell apoptosis and implicated in both Type-1 and Type-2 diabetes. Furthermore, while
369 pancreas destruction causes insulin insufficiency, chronic hyperglycemia is known to negatively affect
370 pulmonary function, by impeding bacterial clearance, increasing oxidative stress and level of pro-

371 inflammatory cytokines, and generating fibrosis in the lungs (48, 49). Importantly, an insufficient Nrf2 activity
372 is often associated with the etiology of diabetes, and has been involved in the development of oxidative
373 stress and inflammatory state in diabetic people (50). Interestingly, NRF2 activation protects mouse model
374 of diabetes from ROS-induced damage (51), suggesting that targeting NRF2-modulated ROS production might
375 show promise in the treatment of pancreas destruction in a context of CF. Thus, having shown promising
376 results in the treatment of diabetes (52), by restoring normal level of inflammation, curcumin might also
377 prevent diabetes in CF.

378 In CF, acute inflammatory responses disrupt airways integrity leading to abnormal tissue remodeling,
379 increased accumulation and deposition of ECM components which can give rise to fibrosis (53). Our findings
380 indicate that curcumin improves tissue repair in CF zebrafish, by reducing tissue damage and fibrotic
381 phenotypes (**Figure 5-6**). The amelioration of tissue integrity is also observed in CF AO, associated with reduction
382 of cell mortality and aberrant tissue remodeling (**Figure 7**), demonstrating that curcumin might prevent lung
383 damage in people with CF. We have not yet identified the molecular events downstream of curcumin treatment
384 in tissue repair and remodeling and how these might regulate collagen deposition. Modulation of macrophage
385 (54) and fibroblast activity (55), two important cell populations in tissue repair processes including collagen
386 deposition, could be plausible cellular targets.

387 Increasing evidence, including our previous works, demonstrates that hyper-susceptibility to infections in CF
388 (56, 57) would be in part linked to defective bactericidal activities in professional phagocytes, themselves directly
389 caused by CFTR dysfunction (58–60). Interestingly, while NRF2 is mostly known to be a regulator of oxidative and
390 inflammatory responses, several studies indicate that NRF2 signaling pathway also plays a critical role in immune
391 defense against pathogens (61). A defect in NRF2 in mice increases susceptibility to several CF-related bacteria,
392 including *P. aeruginosa* (61, 62), *S. aureus* (63) and the mycobacterial species *Mycobacterium avium* (64),
393 suggesting this could account for the infection phenotype in CF. Further investigations are needed to determine
394 how CFTR/NRF2 interactions regulate antibacterial defense and how this defective axis contributes to increased
395 susceptibility to infection in CF.

396 Anti-inflammatory therapies are of particular interest for CF lung disease but must be carefully studied to
397 minimize the risk of impeding host immunity and thus worsening infection, as we recently observed with
398 roscovitine (65). We showed that sulforaphane, another activator of NRF2, increases *M. abscessus* killing in
399 several human models including CF AOs (10, 66), suggesting that activation of NRF2 might be a useful
400 pharmacological approach for CF-associated defects in bacterial clearance. Hypothesizing that a decrease in
401 Nrf2 signaling in people with CF indeed hampers their ability to defend against pathogens, it could be interesting
402 to test whether activation of NRF2 by curcumin restores antimicrobial defense in CF. Moreover, while curcumin
403 inhibits virulence factors in *P. aeruginosa*, such as the formation of biofilm or pyocyanin biosynthesis, gene
404 involved in quorum sensing (67), the antibiotic synergic action of this compound also improves the activity of
405 antibacterial agents against *S. aureus* (68), *M. abscessus* (69) and *P. aeruginosa* (70). Therefore, these results may

406 have significant therapeutic implications for potentially targeting inflammation in CF and improving host immunity
407 to infection.

408 Because the low bioavailability of curcumin, associated with its poor solubility and absorption in free
409 form in the gastrointestinal tract and its rapid biotransformation to inactive metabolites, there is a debate
410 on its effectiveness and utility as a health-promoting compound (41). However, this study, involving
411 inflammation and tissue damage management in CF, was conducted with a free molecule, thus supporting
412 its therapeutic and protective effects. With the availability of highly bioavailable curcumin formulations (71),
413 it should be now possible to exploit the full activities and benefits of curcumin in CF, and clinical trials will be
414 necessary to confirm its effects in humans.

415 To conclude, our findings indicate that rescuing NRF2 using curcumin may be a targeted therapeutic
416 strategy to both mitigate inflammation and restore tissue repair, and thus prevent inflammatory damage in
417 individuals with CF.

418

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432 **Figure legends**

433

434 **Figure 1. Structural analysis of zebrafish Nrf2a and comparison with human NRF2. (A)** Domain organization
435 of human NRF2 (hNRF2), with the target binding partners indicated for each domain, and predicted domain
436 organization for zebrafish Nrf2a. All seven Neh domains seem to be present in Nrf2a. **(B)** 3D-structure of
437 zebrafish Nrf2a predicted with AlphaFold. In this model, long unstructured regions alternate with shorter
438 regions adopting defined secondary structures, mostly α -helices. The Neh domains are represented with the
439 same colour code as in panel A. **(C)** Structural overlay of the AlphaFold-predicted model for zebrafish Nrf2a
440 (light purple) with that of hNRF2 (cyan). The position of the secondary structure elements within the Nrf2a
441 model matches well with those predicted for hNRF2. **(D)** Structural overlay of the Neh1 domains from
442 AlphaFold-predicted Nrf2a structure, from AlphaFold-predicted hNRF2 structure, and from the X-ray
443 structure of the human NRF2-MafG complex (red; PDB ID 7X5F; (20)). The Neh1 domain of Nrf2a aligns well
444 with the corresponding domains of hNRF2, with root mean square deviation (rmsd) values on C α atoms
445 ranging from 0.7 to 2.5 Å². **(E)** Structural overlay of the Neh2 domains from AlphaFold-predicted Nrf2a
446 structure, from AlphaFold-predicted hNRF2 structure, and from the X-ray structure of the hNRF2-Keap1
447 complex (red; PDB ID 3WN7; (21)). The Neh2 domain of Nrf2a aligns well with the corresponding domains of
448 hNRF2, with rmsd values on C α atoms ranging from 2.5 to 4.0 Å².

449

450 **Figure 2. Injury-induced inflammatory responses are exacerbated in the absence of Nrf2 signaling in**
451 **zebrafish larvae. (A)** Schematic of aseptic injury-induced inflammation assay and analyses. Caudal fin
452 amputations (dotted lined) were performed proximally through the notochord (N) at 3 days post-fertilization
453 (dpf). **(B)** Control MO and *cftr* MO (generated using the validated *cftr* morpholino (16)) were caudal fin
454 amputated then the level expression of mRNA for *nrf2a* (left) and *nrf2b* (right) genes are evaluated at 2 hours
455 post-amputation (hpA). Graphs showing the fold change over total tissue from uninjured larvae (relative gene
456 expression from at least 3 independent experiments performed in triplicates, mean and quartiles shown,
457 Two-tailed Student *t*-test). **(C–F)** Neutrophil mobilization to tissue injury in the neutrophil-specific
458 *TgBAC(mpx:EGFP)i114* line (22) is observed and enumerated at 2 hpA under a fluorescence microscope. The
459 neutrophil count area is defined as the region between the blood vessels (BV) end and the amputation edge
460 (AE). **(C)** Number of neutrophils mobilized to the wound in control MO, *nrf2a* MO, *nrf2b* MO or double *nrf2a*
461 MO / *nrf2b* MO (*nrf2a+b* MO). Each dot represents the number of neutrophils at the wound in a single larva
462 (3 independent experiments, One-way ANOVA with Dunnett's comparisons test). **(D)** Representative images
463 of injured tails (scale bars, 100 μ m). The images are denoted as green data points in (C). **(E)** Inhibition of
464 *nrf2a*, *nrf2b* or *nrf2a+b* was carried out in control and CF animals by injecting the *nrf2a* morpholino, the *nrf2b*
465 morpholino or the *nrf2a* + *nrf2b* morpholinos reciprocally. Neutrophil recruitment assay (3 independent
466 experiments, Two-way ANOVA with Tukey's multiple comparisons test). **(F)** Control MO and *cftr* MO were
467 treated with the NRF2 inhibitor ML 385, the NRF2 agonist CDDO-Me or DMSO (as mock control) prior to

468 caudal fin amputation procedure, then injured and immediately put back in treatments. Neutrophil number
469 at injured tails were enumerated at 2 hpA (3 independent experiments, Two-way ANOVA with Tukey's
470 multiple comparisons test). **(G)** Relative expression of mRNA for *duox*, *nqo1*, *hmx1*, *cxcl8* and *il1 β* at 2 hpA
471 (*duox*, *cxcl8* and *il1 β*) or 8 hpA (*nqo1* and *hmx1*) from at least 3 independent experiments performed in
472 triplicates (mean and quartiles shown, Two-tailed Student *t*-test).

473

474 **Figure 3. Nrf2a-depleted zebrafish exhibit reduced tissue repair responses after injury. (A-I)** Tissue repair
475 performance assessment. Caudal fin amputations were performed without injury to the notochord (dotted
476 line) at 2 dpf, then the potential of tissue repair was evaluated at 2 **(E-I)** or 3 **(B-D)** days post-amputation
477 (dpA). **(B-C)** Control MO and *nrf2a* MO were caudal fin amputated. Representative imaging of injured tail fin
478 (scale bars, 100 μ m) **(B)** and measurement of regenerated fin areas, lengths and widths **(C)** (n=14-18 from 3
479 independent experiments, Two-tailed Student *t*-test). Error bars represent standard error of the mean (SEM).
480 The regenerated fin areas were defined as the region between the amputation edge and the end of the
481 extended fin. The regenerated fin lengths were measured from the notochord tip to the end of the extended
482 fin. The regenerated fin widths were defined as the max width measured from the amputation edge to the
483 end of the extended fin. **(D)** Inhibition of *nrf2a* was carried out in WT and CF animals by injecting the *nrf2a*
484 morpholino. Measurement of regenerated tail fin areas, lengths and widths in the presence or absence of
485 *nrf2a* (n=16 from 3 independent experiments, Two-way ANOVA with Tukey's multiple comparisons test). **(E)**
486 Second harmonic generation (SHG) and high magnification bright field imaging showing epithelial projection
487 associated with collagen fibers (scale bar, 10 μ m). **(F)** Representative bright field microscopy of injured caudal
488 fin showing extended collagen fiber-containing epithelial projections (arrows) pushing the healing plane
489 forward (scale bar, 100 μ m). Morpholino knockdown of *nrf2* expression reduced the appearance of
490 projections, suggesting that Nrf2a modulates the formation of collagen fiber in zebrafish. **(G-H)** Proportion
491 of larvae forming visible projections **(G)**; n=14-16, Fisher test) and the mean \pm SEM number of projections per
492 larvae **(H)**; n=14-16, Two-way ANOVA, Tukey's multiple comparisons test) **(I)** Measurement of wound contour
493 length in the presence or absence of *nrf2a* (n=14-18 from 3 independent experiments, Two-way ANOVA with
494 Tukey's multiple comparisons test).

495

496 **Figure 4. NRF2 activation by curcumin normalizes inflammation in *cftr*-depleted zebrafish. (A)** Control MO
497 and *cftr* MO were pre-treated with DMSO (as control) or curcumin before injury, then injured and
498 immediately put back in treatments until analysis. **(B)** mRNA levels of *nrf2a* at 2 hpA (relative gene expression
499 from at least 3 independent experiments performed in triplicates, Two-way ANOVA with Tukey's multiple
500 comparisons test). **(C)** Neutrophil number at the wound at 2 hpA (3 independent experiments, Two-way
501 ANOVA with Tukey's multiple comparisons test). **(D)** Representative images of amputation-induced
502 neutrophil mobilization in *cftr*-MO treated or not with curcumin (scale bars, 100 μ m). **(E)** Inhibition of *nrf2a*
503 was carried out in CF animals by injecting the *nrf2a* morpholino. Neutrophil number at the wound at 2 hpA

504 (3 independent experiments, Two-way ANOVA with Tukey's multiple comparisons test). **(F)** Schematic
505 illustration of laser-mediated injury assay triggering ROS production by epithelial cells. **(G-H)** *cftr* MO are
506 stained with CellROX[®], treated, laser-injured, then ROS production was time-lapse imaged and quantified
507 over 90 minutes by confocal microscopy. **(G)** ROS intensity at the wound site over the time course of
508 inflammation. Line of best fit shown is calculated by linear regression. *P*-value shown is for the difference
509 between the 2 slopes (n=12, performed as 3 independent experiments). **(H)** Pseudocolored imaging of laser-
510 injured caudal fin showing representative ROS production throughout inflammation. **(I-M)** mRNA levels of
511 *duox*, *nqo1*, *hmox1*, *cxcl8* and *il1 β* at 2 hpA (*duox*, *cxcl8* and *il1 β*) or 8 hpA (*nqo1* and *hmox1*) (relative gene
512 expression from at least 3 independent experiments performed in triplicates, Two-way ANOVA with Tukey's
513 multiple comparisons test). **(N-O)** *cftr* MO *Tg(il1b:eGFP-F)ump3* (72) were caudal fin amputated then the
514 expression of *Il1 β* was observed and analyzed. Relative *Il1 β* intensity at 2hpA in *cftr* MO treated or not with
515 curcumin (n=18 from 3 independent experiments; Mann-Whitney U test) **(N)** and associated pseudocolored
516 photomicrographs of injured tails **(O)** revealing *il1 β* expression at the wound-edge (scale bar, 100 μ m).

517

518 **Figure 5. Curcumin improves defective tissue repair capacity in Cftr-depleted animals. (A-C)** Control MO
519 and *cftr* MO were treated with DMSO or curcumin prior to caudal fin amputation procedure, then injured
520 and immediately put back in treatments for 4 h. The potential of tissue repair was evaluated at 72 hpA. **(B)**
521 Brightfield microscopy of injured caudal fin showing tissue regeneration in *cftr* MO treated with curcumin
522 versus DMSO (scale bars, 100 μ m). **(C)** Measurement of regenerated caudal fin areas, lengths and widths
523 following compounds exposure (n=20, Two-way ANOVA, Tukey's multiple comparisons test).

524

525 **Figure 6. Curcumin reduces tissue damage by promoting remodeling of collagen fibers in CF zebrafish.**
526 Control MO and *cftr* MO were pre-treated with DMSO or curcumin prior to tail fin amputation procedure,
527 then injured and immediately put back in treatments for 4 h. SHG microscopy of the regenerated tail fin was
528 performed at 2 **(A-D)** or 3 dpA **(E-N)**. **(A)** surface rendering of a 3D reconstruction showing the tissue wound
529 edge in the bright field and corresponding SHG imaging Z projections to reveal collagen projections in injured
530 tails in control versus CF animals (scale bars, 25 μ m). **(B-C)** Proportion of larvae forming projections **(B;** n=20,
531 Fisher test) and the number of projections per larvae **(C;** n=20, Two-way ANOVA, Tukey's multiple
532 comparisons test), in fishes treated or not with curcumin. **(D)** Measurement of wound contour length in fishes
533 treated or not with curcumin (n=20 from 3 independent experiments, Two-way ANOVA with Tukey's multiple
534 comparisons test). **(E)** 3D surface-rendered reconstruction of collagen fibers in regenerated tails to illustrate
535 the spatial organization of fiber relative to regenerated tissue with tissue wound edge in the bright field and
536 corresponding SHG imaging Z projections (scale bars, 100 μ m). **(F)** Areas of collagen fibers in Control MO
537 versus *cftr* MO regenerated fin as a function of tissue areas (left), and areas of collagen fibers in the
538 regenerated fin of CF fishes following DMSO or curcumin exposure as a function of tissue areas (right). **(G)**
539 Graph showing the ratio of area devoid of SHG fibers (from fiber ends to wound edge) following fin

540 amputation. Ratios were determined by measuring the areas devoid of SHG fibers normalize with total
541 regenerated tissue from F. **(H)** CT-FIRE-generated projections of SHG imaging of collagen fibers in the tail fin
542 showing changes in organization of collagen fibers during tissue repair process. **(I-L)** Graphs showing
543 quantitation of fiber number **(I)**, length **(J)**, width **(K)** as determined using CT-FIRE fiber analysis software (3
544 independent experiments, Two-way ANOVA with Tukey's multiple comparisons test). **(L)** mRNA levels of *tgf-*
545 *β* at 3dpA (relative gene expression from at least 3 independent experiments performed in triplicates, Two-
546 way ANOVA with Tukey's multiple comparisons test). **(M)** Graphs showing quantitation of fiber angle as
547 determined using CT-FIRE fiber analysis software (3 independent experiments, Two-way ANOVA with Tukey's
548 multiple comparisons test). **(N)** 3D reconstruction from fin in cross section view revealing the spatial
549 organization between tissue and the fibers (scale bars, 100 μm), with fibers found within the space between
550 the 2 layers.

551

552 **Figure 7. Treatment with curcumin alleviates inflammatory state and tissue damage in human CF airway**
553 **organoid. (A-C)** Healthy or CF airway organoid (AO) were incubated with vehicle (DMSO) control or 30 μM
554 curcumin for 1 day. Gene expression levels of *NRF2* **(A)**, the pro-inflammatory cytokines *IL8* and *IL1β* **(B)**, the
555 oxidative stress-related gene *DUOX1* and the *NRF2*-activated genes NQO1 and HMOX1 **(D)** were determined
556 by RT qPCR (relative gene expression from at least 3 independent experiments performed in triplicates,
557 ANOVA with Tukey's multiple comparisons test). **(D-E)** Cell death analysis in AO treated with vehicle (DMSO)
558 control or 30 μM curcumin for 4 days. Mean fluorescence intensity quantification of the propidium iodide
559 (PI) incorporation **(D)** and representative images (scale bars, 300 μm) **(E)** in healthy and CF organoids treated
560 or not with curcumin (n=12-13, from 3 independent experiments, Two-way ANOVA with Tukey's multiple
561 comparisons test). Data for at least three independent experiments are expressed as violin plots. **(F-G)**
562 Quantification of the epithelial thickness (n=12-13, from 3 independent experiments, ANOVA with Tukey's
563 multiple comparisons test) **(F)** and representative images (scale bars, 300 μm) **(G)** in healthy and CF organoids
564 treated or not with 30 μM of curcumin for 4 days.

565

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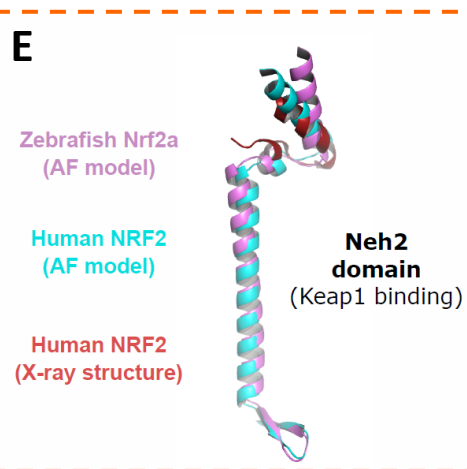
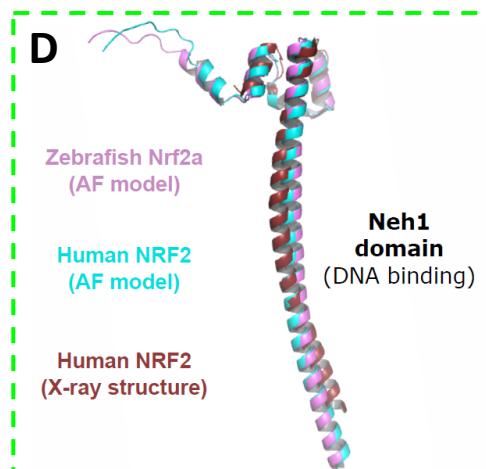
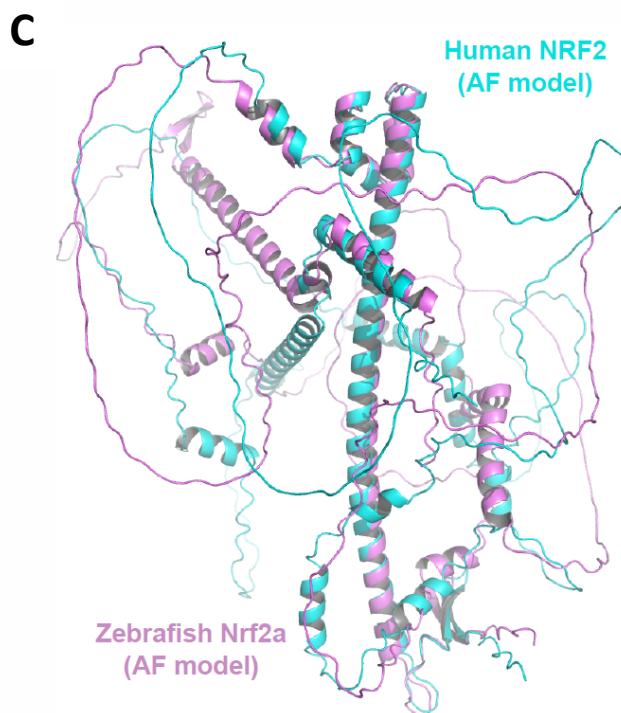
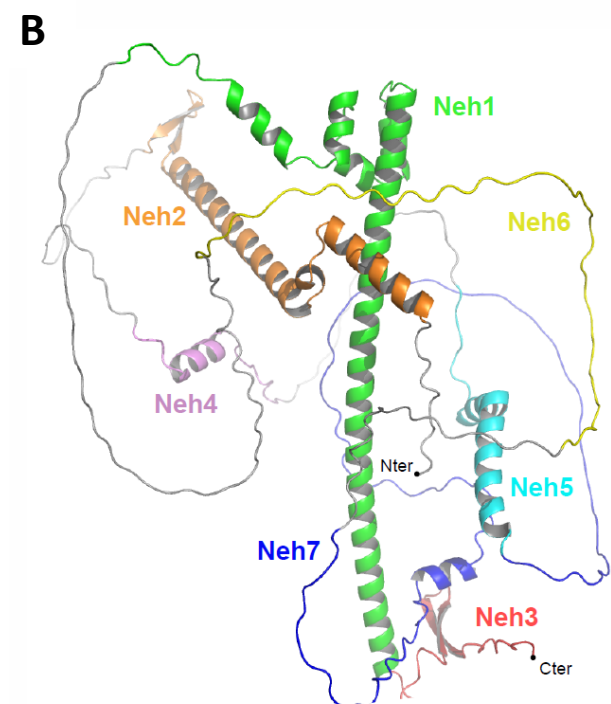
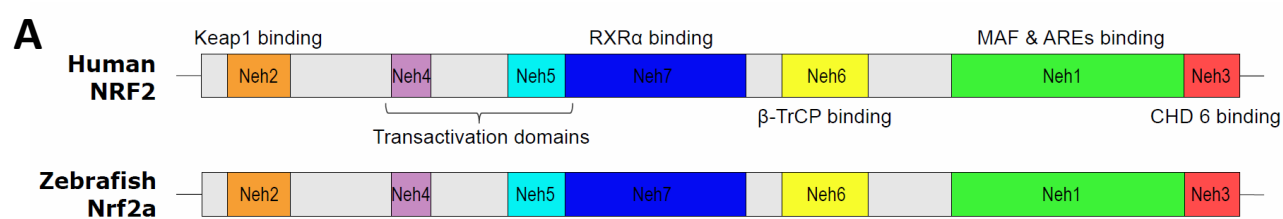


Fig 1

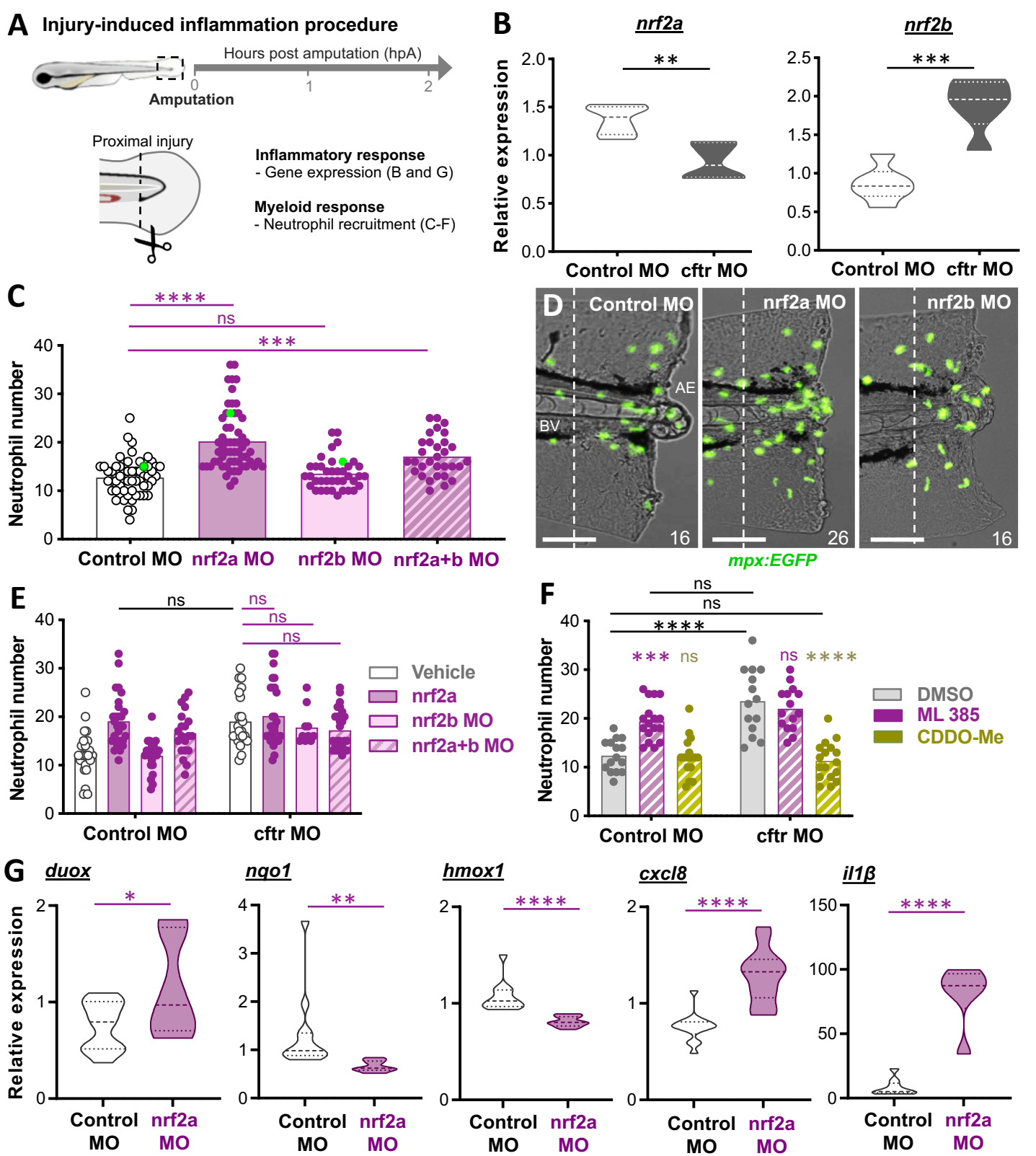


Fig 2

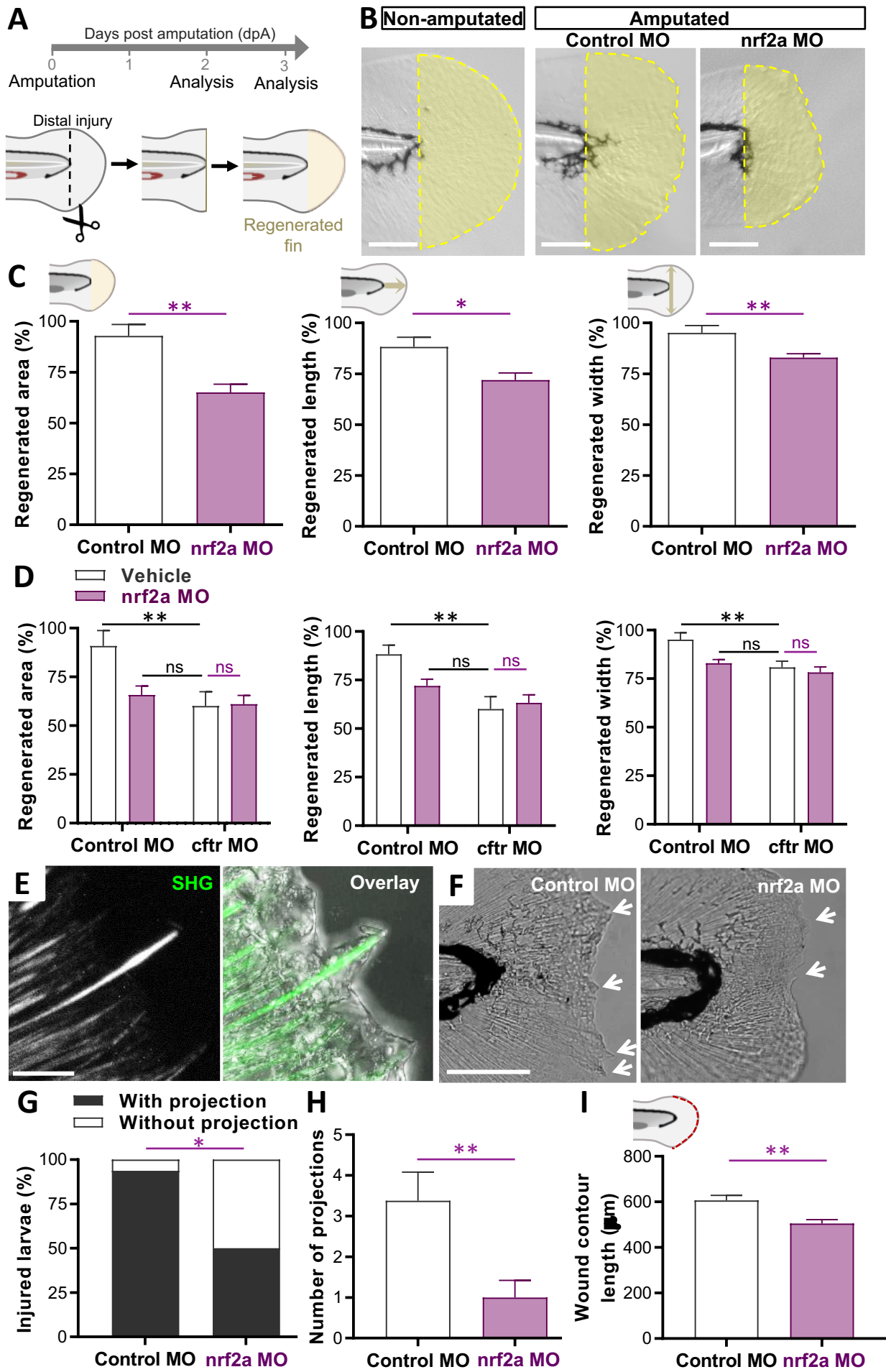


Fig 3

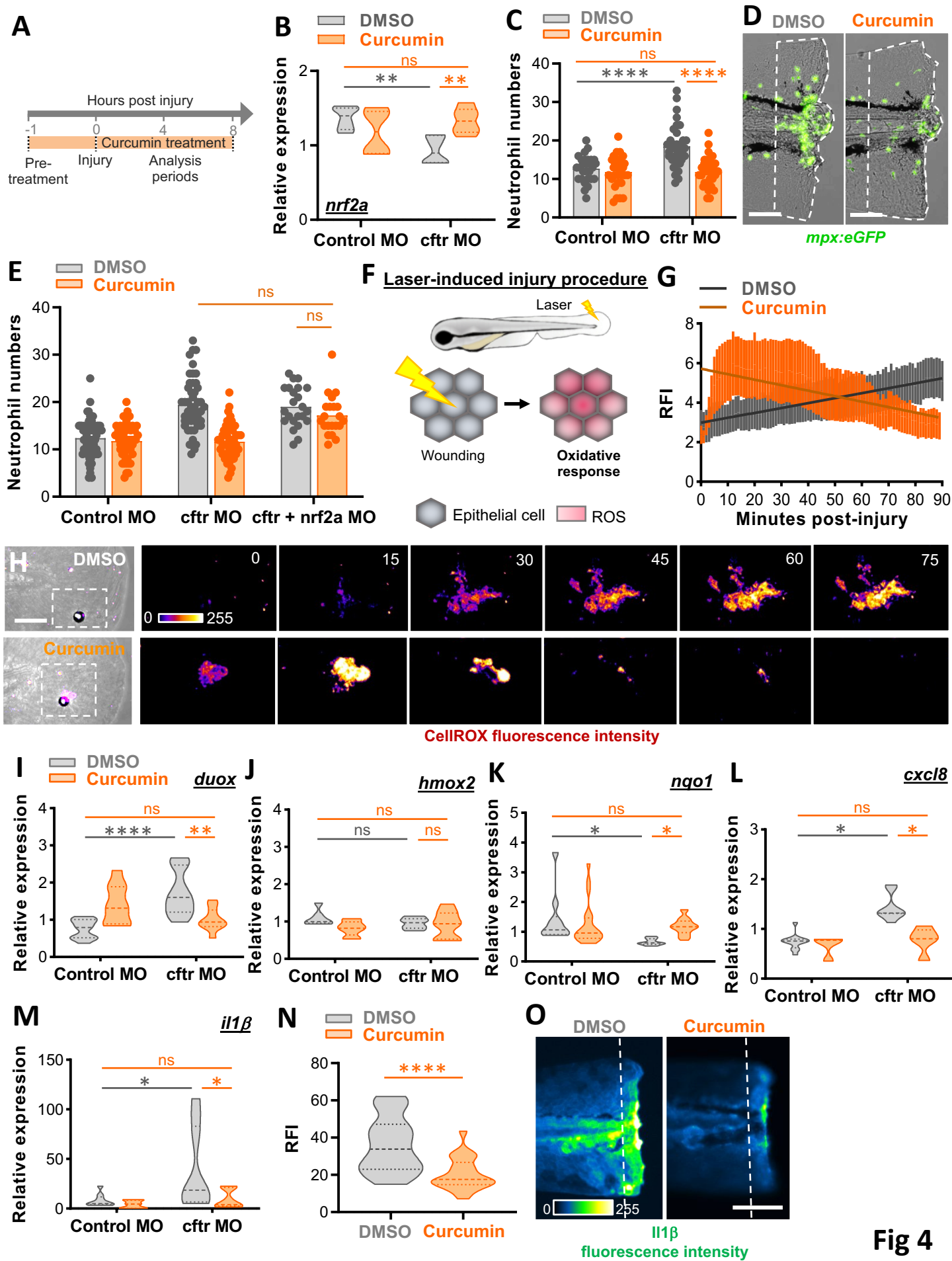


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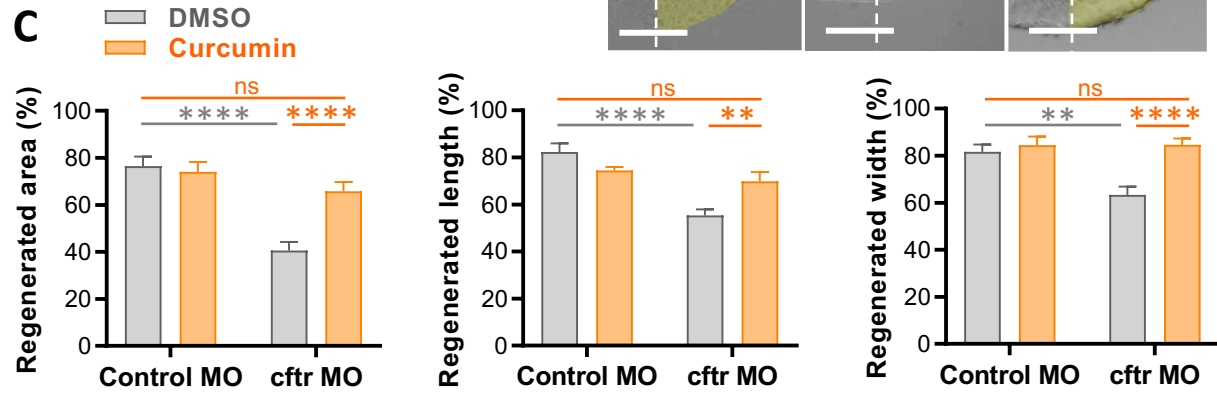
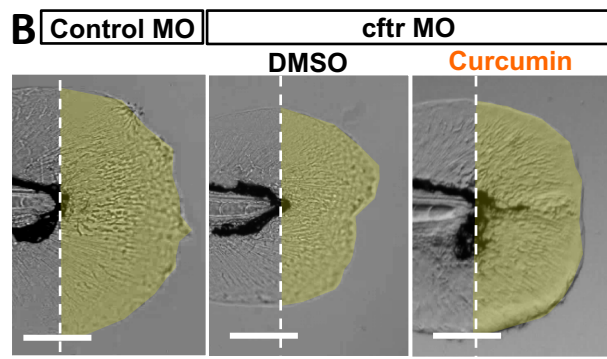
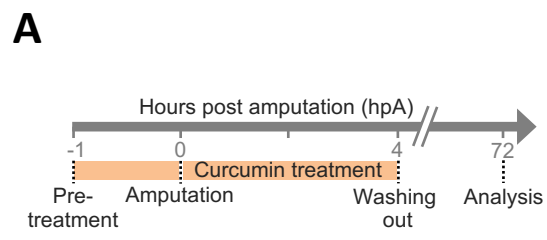


Fig 5

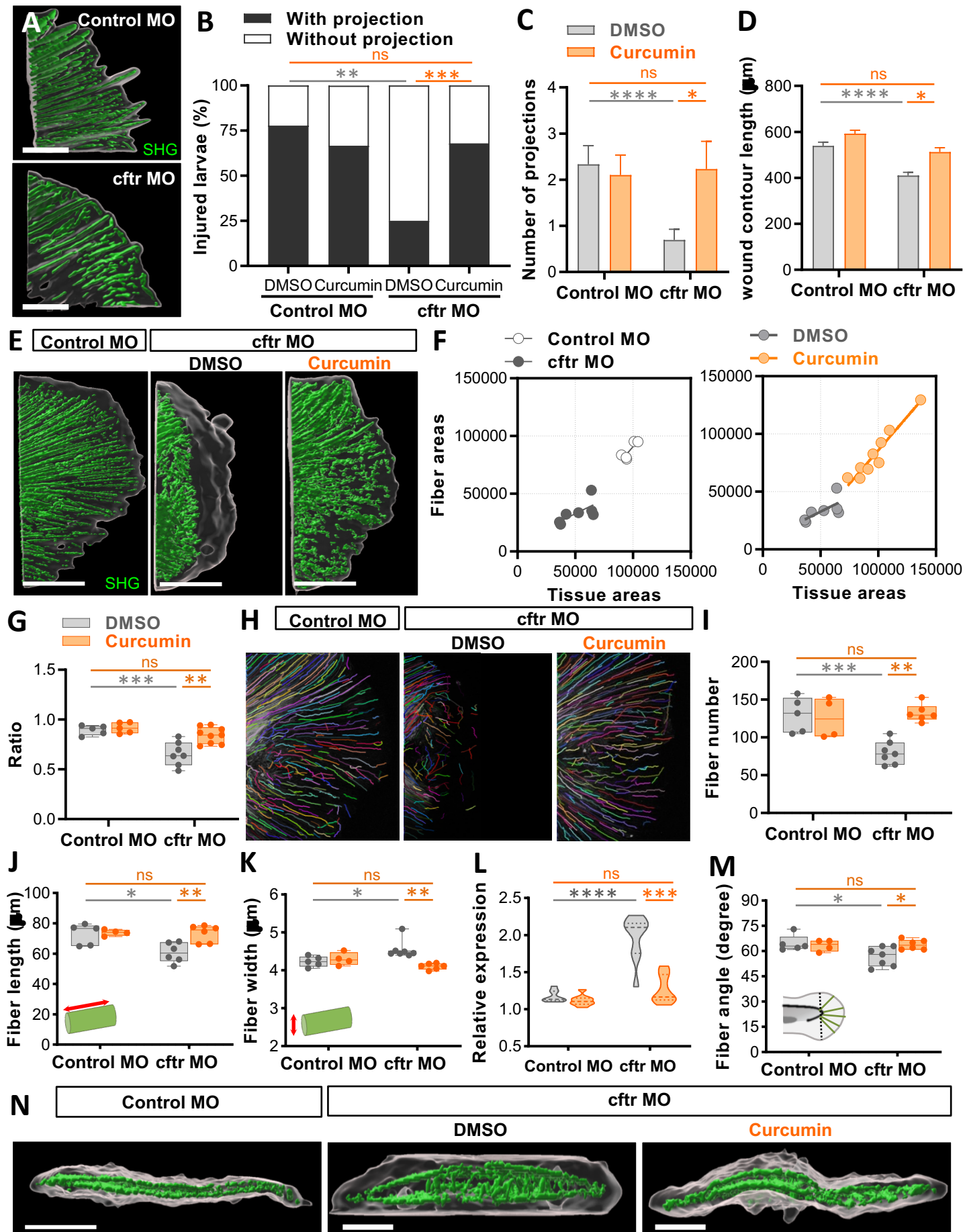


Fig 6

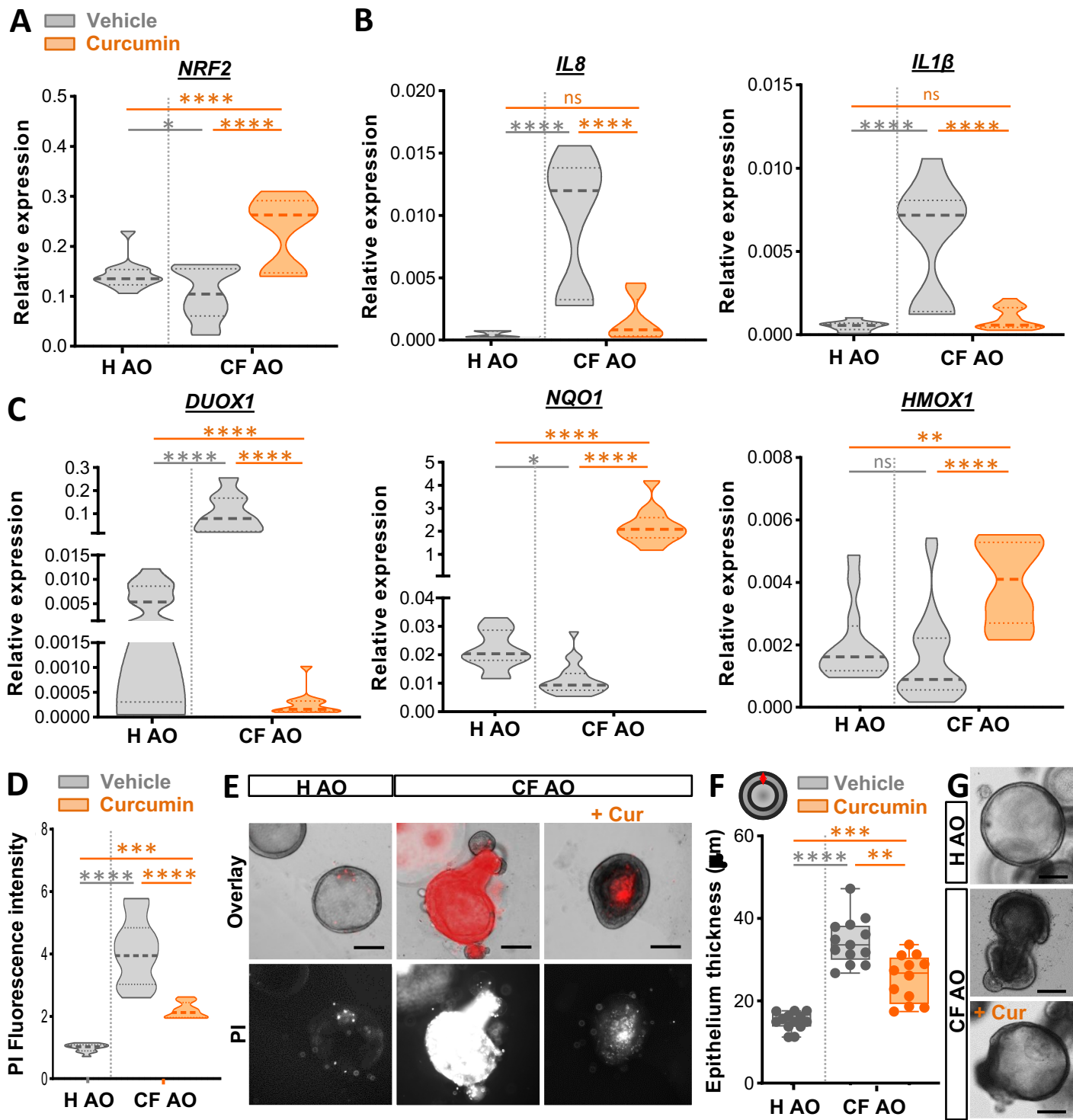


Fig 7