

1 **Hydroxylases regulate intestinal fibrosis through the suppression**
2 **of ERK mediated TGF- β 1 signaling**

3 ^{1,2}Mario C. Manresa, ³Murtaza M. Tambuwala, ⁴Praveen Rhadakrishnan, ⁴Jonathan M.
4 Harnoss, ¹Eric Brown, ^{1,5}Miguel A. Cavadas, ¹Ciara E. Keogh, ^{1,5}Alex Cheong, ⁶Kim E.
5 Barrett, ¹Eoin P. Cummins, ⁴Martin Schneider and ^{1,5}Cormac T. Taylor.

6 ¹School of Medicine and Medical Science, UCD Conway Institute, University College
7 Dublin, Belfield, Dublin 4, Ireland. ²School of Medicine and Medical Science, Charles
8 Institute of Dermatology, University College Dublin, Belfield, Dublin 4, Ireland. ³School of
9 Pharmacy and Pharmaceutical Science, Ulster University, Coleraine, County Londonderry
10 BT52 1SA, Northern Ireland. ⁴Department of General, Visceral and Transplantation Surgery,
11 University of Heidelberg, Im Neuenheimer Feld 110, Heidelberg, 69120, Germany. ⁵Systems
12 Biology Ireland, University College Dublin, Belfield, Dublin 4, Ireland. ⁶Department of
13 Medicine and Biomedical Sciences Ph.D. Program, University of California, San Diego,
14 School of Medicine, La Jolla, CA 92093, USA

15 **Running head:** Hydroxylase inhibition suppresses fibrosis

16 **Corresponding author:** Cormac T Taylor, UCD Conway Institute, University College
17 Dublin, Belfield, Dublin 4, Ireland. Email: Cormac.Taylor@UCD.ie. Tel: +353 1-716-673.
18 Fax: +353 1-716-6701.

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20

21 **Abstract**

22 Fibrosis is a complication of chronic inflammatory disorders such as
23 inflammatory bowel disease (IBD), a condition which has limited therapeutic
24 options and often requires surgical intervention. Pharmacologic inhibition of
25 oxygen-sensing prolyl hydroxylases (PHD), which confer oxygen-sensitivity
26 upon the hypoxia inducible factor (HIF) pathway, has recently been shown to
27 have therapeutic potential in colitis, although the mechanisms involved remain
28 unclear. Here, we investigated the impact of hydroxylase inhibition on
29 inflammation-driven fibrosis in a murine colitis model. Mice exposed to dextran
30 sodium sulfate followed by period of recovery developed intestinal fibrosis
31 characterized by alterations in the pattern of collagen deposition and infiltration
32 of activated fibroblasts. Treatment with the hydroxylase inhibitor
33 dimethyloxalylglycine (DMOG) ameliorated fibrosis. TGF- β 1 is a key regulator
34 of fibrosis which acts through the activation of fibroblasts. Hydroxylase
35 inhibition reduced TGF- β 1-induced expression of fibrotic markers in cultured
36 fibroblasts suggesting a direct role for hydroxylases in TGF- β 1 signalling. This
37 was at least in part due to inhibition of non-canonical activation of extracellular
38 signal-regulated kinase (ERK) signalling. In summary, pharmacologic
39 hydroxylase inhibition ameliorates intestinal fibrosis, through suppression of
40 TGF- β 1-dependent ERK activation in fibroblasts. We hypothesize that in
41 addition to previously reported immunosuppressive effects, hydroxylase
42 inhibitors independently suppress pro-fibrotic pathways.

43 **New and noteworthy**

44 Here we show that hydroxylase inhibitors reduce fibrosis associated with
45 intestinal inflammation in vivo. At the cellular level, our data suggest a new
46 HIF independent role of hydroxylase inhibition in the regulation of TGF- β 1
47 signalling. These data also suggest that non-canonical ERK signalling pathway
48 is regulated by hydroxylase inhibition. Together, our results show the
49 therapeutic potential of hydroxylase inhibitors for the treatment of intestinal
50 fibrosis.

51 **Keywords**

52 Hypoxia; Inflammatory bowel disease; Intestinal fibrosis; Hydroxylase
53 inhibition; Transforming growth factor β 1 (TGF- β 1) signalling.

54

55 **Introduction**

56 Intestinal fibrosis is a debilitating complication of inflammatory bowel disease
57 (IBD)(16, 19, 58). Most approaches to therapeutic intervention in IBD are
58 focused on the control of inflammation rather than fibrosis(58). However,
59 surgical intervention is commonly necessary due to the formation of fibrotic
60 tissue(58, 63). Therefore, there is an unmet clinical need for therapies that
61 suppress pro-fibrotic pathways in IBD.

62 Fibrosis occurs as a result of an overactive wound healing response and is
63 characterized by excessive deposition of extracellular matrix (ECM)(16, 47,
64 58). Fibrosis is a complication of multiple chronic inflammatory disorders
65 including chronic kidney disease(12, 55), interstitial lung disease(1, 79) and
66 chronic liver disease(38, 50) as well as IBD(16, 19, 81). At a cellular level,
67 fibrosis occurs as the result of the over-activation of fibroblasts and other ECM-
68 producing cells(1, 12, 16, 79). Among the factors regulating fibrosis,
69 transforming growth factor- β 1 (TGF- β 1) is the key regulator of healing
70 responses that is implicated in most fibrotic disorders(10, 16, 19, 38). The
71 interaction of TGF- β 1 with its cognate receptors on fibroblasts activates
72 canonical (Smad-mediated) and non-canonical (Mitogen Activated Protein
73 Kinases (MAPK)-mediated) signalling pathways that lead to the differentiation
74 of fibroblasts into pro-fibrotic myofibroblasts(10). Through these pathways,
75 TGF- β 1 induces the expression of crucial pro-fibrotic genes such as α -smooth

76 muscle actin (α -SMA), collagen and matrix metalloproteinases and stimulates
77 fibroblast migration to wounded tissue.

78 Hydroxylases are oxygen sensing enzymes which confer hypoxic sensitivity
79 upon the hypoxia inducible factor (HIF) pathway. Four HIF hydroxylases have
80 been identified to date. Three of these are prolyl hydroxylases (termed PHD1-3)
81 which regulate HIF stability, and the fourth is an asparagine hydroxylase,
82 termed factor inhibiting HIF (FIH), that controls HIF transcriptional activity.
83 The PHD2 isoform is the main regulator of HIF stability through targeting HIF-
84 α subunits for hydroxylation-dependent proteosomal degradation(7). These
85 enzymes have also been identified as key players in inflammatory responses(13,
86 20, 53, 61). Hydroxylase inhibitors have recently been found to be well
87 tolerated in patients in clinical trials for efficacy in anemia(9, 28). These
88 compounds have also been found to ameliorate inflammation in multiple animal
89 models of colitis(14, 15, 22, 36, 60, 64). However, it remains unclear whether
90 the protective effects of these drugs extend to the amelioration of intestinal
91 fibrosis. Furthermore, it is also unclear if their protective effects in colitis are
92 dependent on the regulation of HIF or other pathways(36, 60, 61, 64). Moreover
93 recent studies have highlighted a potentially important role for PHD-2 in the
94 regulation of wound healing responses(32, 78, 80).

95 In this study, we investigated the effects of hydroxylase inhibition on the
96 development of intestinal fibrosis. Developing our understanding of the

97 mechanisms underpinning the protective effects of hydroxylase inhibitors in
98 intestinal inflammatory disease will enhance our understanding of their
99 therapeutic potential.

100

101 **Materials and methods**

102 *1. Animal studies*

103 C57BL/6 mice were obtained from Charles River U.K. Prolyl hydroxylase-2
104 heterozygous knockout mice (PHD2+/-) of the Swiss129 background were
105 provided by Professor Martin Schneider (University of Heidelberg, Germany).
106 These mice have been characterised and used in several previous studies(37,
107 48). All *in vivo* experiments were performed in compliance with regulations of
108 the Irish Department of Health and approved by the University College
109 Dublin's animal research ethics committee or approved by the ethical
110 commission of the local government (No. G263/14) and carried out at the
111 Interfaculty Biomedical Faculty, University of Heidelberg. All experiments
112 were carried out according to the federation of laboratory animal Science
113 association (FELASA) guidelines. Female and male mice (ages 10-12 weeks)
114 were used. For induction of fibrosis, mice were exposed to 2.5% or 5% DSS
115 (MP Biomedicals, Solon, OH, U.S.A) in drinking water for five days. On day 6
116 mice were switched to normal drinking water and allowed to recover for 8 or 14
117 days as indicated. During this period, mice were treated with 8mg/mouse
118 DMOG (i.p) every 48 hours.

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121 *2. In vitro experiments*

122 Primary human colonic fibroblasts (CCD-18Co) were purchased from the
123 American Type Culture Collection (ATCC, LGC Standards, Middlesex, UK).
124 Immortalized mouse embryonic fibroblasts (MEF) were a kind gift from Dr
125 Alexander Hoffmann (University of California, Los Angeles, U.S.A). Primary
126 MEF of both wild type (WT) and PHD-2^{+/-} backgrounds were isolated from
127 murine foetuses of the appropriate background. All cells were grown in 1X
128 Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham,
129 MA, U.S.A) containing 4.5 g/l glucose and L-glutamine. The medium was
130 supplemented with 10% foetal bovine serum, 50u/ml penicillin and 50 mg/ml
131 streptomycin. In the case of primary MEFs, the medium was also supplemented
132 with 2mM L-glutamine. For CCD-18Co cells, 0.1mM non-essential amino acids
133 were added. For all experiments, cells were switched to serum free medium 24
134 hours prior to treatment. All treatments were performed in serum free medium.
135 Cells were stimulated with TGF- β 1 for the indicated time periods to induce
136 expression of fibrotic markers and activation of TGF- β 1-stimulated signalling
137 pathways. To investigate the effects of hydroxylase inhibition on TGF- β 1
138 mediated responses, cells were treated with DMOG or JNJ1935 for 1 or 8 hours
139 prior to TGF- β 1 stimulation as indicated. Hypoxia was achieved in a hypoxia
140 chamber (Coy laboratories, Grass Lake, MI, USA). Steady-state atmospheric
141 levels of oxygen inside the chamber were reduced to 1%, at a temperature of

142 37°C with 5% CO₂ (balance N₂) in humidified conditions. Media added to cells
143 during hypoxic exposures was pre-equilibrated to hypoxia overnight. For
144 studies where the effects of hydroxylase inhibitors were compared to MEK
145 inhibitors, cells were treated with the MEK inhibitor PD186416 for 1 hour prior
146 to TGF-β1 stimulation. For studies assessing the involvement of HIF in
147 hydroxylase inhibitor mediated effects on fibrosis, cell were treated with
148 Digoxin or Camptothecin and then treated with DMOG prior to TGF-β1
149 stimulation.

150 *3. Estimation of disease parameters and histologic analysis*

151 Body weight, presence of blood in feces, and stool consistency/diarrhea were
152 recorded daily for each mouse to determine the disease activity index (DAI).
153 These parameters were scored as previously described(64). On termination of
154 the experiments, mice were sacrificed by cervical dislocation and approximately
155 1cm from the distal colon was collected, fixed in 10% formalin and embedded
156 in paraffin. 4 μm sections were cut, mounted on slides, deparaffinized with
157 xylene, and rehydrated in a graded series of alcohols. For assessment of tissue
158 inflammation, sections were stained using hematoxylin and eosin (H&E).
159 Images were taken using an Aperio scanscope XT (Aperio technologies, Vista,
160 CA, U.S.A). Tissue inflammation was assessed in a blinded fashion by 2-3
161 independent experienced observers and expressed as an average between the
162 estimations provided. The score was calculated as previously described(61).

163 Fibrosis was assessed as changes in the amount and pattern of collagen
164 deposition in the colonic mucosa and submucosa using Picrosirius red staining
165 (0.5g of direct red 80 (Sigma) and 0.5g of fast green (Sigma) in 500 ml of
166 saturated picric acid solution) and images obtained as described above. Tissue
167 collagen was quantified using ImageJ (National Institutes of Health, Bethesda,
168 MD) and a quantification method developed in-house. Briefly, images were
169 separated into different colour channels and the red channel was selected for
170 quantification of Collagen. From images obtained using double polarized
171 microscopy, collagen-I was quantified, whereas from images obtained using
172 transmitted light, the total amount of collagen was quantified. The colour
173 threshold was adjusted to obtain black and white images, where collagen
174 structures appeared in black on a white background. A small square fitting the
175 width of the sub-mucosal area was drawn and the percentage of black vs white
176 was quantified in 6 random areas of the sub-mucosa for each imaged sample.
177 Alternatively, the lamina propria was separated using a drawing tool and total
178 mucosal collagen quantified as the percentage of black over a white
179 background.

180 To analyse the presence of activated fibroblasts in the colon,
181 immunofluorescent staining for α -SMA was performed using 4', 6-diamidino-2-
182 phenylindol (DAPI) to counterstain nuclei (details on the primary and secondary
183 antibodies can be found in table 1, antibodies list). Quantification of α -SMA

184 was developed using the total percentage of area tool in ImageJ[®] using a method
185 similar to that described for collagen quantification. Briefly, images were split
186 into color channels and the green channel was selected. The threshold of the
187 signal was adjusted and color filter applied so the green signal appeared as
188 black versus a white background. The total amount of black signal in the
189 mucosal area was quantified using ImageJ.

190 For assessment of the presence of phospho-ERK positive cells in fibrotic
191 tissues, immunohistochemistry was performed using a specific phospho-ERK
192 primary antibody (details can be found in table 1). The procedure was
193 developed using a Vectastain Elite ABC-kit (Vector Laboratories, CA, U.S.A)
194 following manufacturer's instructions with 3, 3'-Diaminobenzidine (DAB) used
195 as substrate. Haematoxylin was used to counter-stain nuclei.

196 *4. Wound healing assay*

197 MEF cells were grown to confluence in 6 well plates. Once confluent, cells
198 were starved overnight in serum free medium. Monolayers were treated with
199 DMOG for 1 hour. After treatment, monolayers were scratched with a p200 tip
200 to produce a wound, and stimulated with TGF-beta for 24 hours. Cells were
201 then fixed in 4% p-formaldehyde and stained for alpha-SMA as described in
202 the previous section.

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204 *5. Western blotting*

205 Whole cell protein lysates preparation and western blot analysis were performed
206 according to previously described methods(15). A full list of the antibodies used
207 can be found in table 1.

208 *6. Quantitative real time polymerase chain reaction*

209 RNA was isolated using trizol based extraction. Complementary DNA was
210 synthesized using standard protocols and quantitative real time polymerase
211 chain reaction carried out as previously described(20). A full list of targets
212 analysed and primers used can be found in table 2.

213 *7. luciferase reporter studies*

214 All transfections were performed following previously described methods(52).
215 For the study of the effects of hydroxylase inhibition on TGF-beta mediated
216 activation of Smad responses, MEF cells were transfected with the luciferase
217 reporter SBE4-Luc which was a gift from Bert Vogelstein (addgene
218 plasmid#16495)(75). To validate the specificity of the reporter, MEF were co-
219 transfected with SBE4-Luc and Smad2 or Smad3 overexpression constructs,
220 and the activation of the luciferase reporter was evaluated. pCMV5B-Flag-
221 Smad3 was a gift from Jeff Wrana (addgene pasmid #11742)(40). pCMV5
222 Smad2-HA was a gift from Joan Massague (addgene plasmid #14930)(25).

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224 *8. Statistical analysis*

225 Graph Pad Prism version 5.0 was used for all statistical analysis. One-way
226 ANOVA followed by a Newman Keuls post-test was used for comparison of
227 multiple groups. Student's t-test was used for individual group comparisons.
228 Differences were considered statistically significant when the p-value was
229 ≤ 0.05 . There was a minimum of 3 experimental replicates per group.

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242 **Results**

243 *1. DMOG reduces intestinal fibrosis in DSS-induced colitis*

244 Hydroxylase inhibitors have been shown to have beneficial effects in multiple
245 models of experimental colitis although their effect on colitis-associated fibrosis
246 remains unknown(15, 60, 64). Figure 1A shows representative images of mouse
247 colon stained with H&E (reflecting tissue inflammation-upper panels), picro-
248 sirius red (reflecting collagen deposition-middle panels) or with
249 immunofluorescence staining for α -SMA (reflecting fibrotic fibroblast
250 infiltration-lower panels). DSS-treatment led to mucosal inflammation,
251 disruption of intestinal structures and submucosal edema which partially
252 recovered following 14 days of natural recovery (Figures 1A). Treatment with
253 the hydroxylase inhibitor DMOG reduced markers of inflammation and
254 significantly accelerated the recovery of disease activity scores (figures 1A, 1B
255 and 1C). Inflammation was associated with the development of fibrosis,
256 reflected by a change in the pattern of collagen deposition characterized
257 particularly by increased submucosal collagen (Figures 1A and 1D, white
258 arrows point areas of higher collagen deposition). A similar pattern of collagen
259 deposition in the submucosa in a model of DSS induced fibrosis has previously
260 been described by Ding and colleagues(17). Fibrosis was also characterized by
261 the presence of infiltrating α -SMA positive fibroblasts in the colonic mucosa
262 (Figures 1A and 1E, white arrows point areas of fibroblast infiltration). Mice
263 treated with DMOG demonstrated reduced inflammation with normal mucosal

264 structures, an absence of inflammatory infiltrates and a reduction in submucosal
265 edema (figure 1A and 1C). DMOG treatment also reduced and normalized the
266 pattern of submucosal collagen deposition, and reduced infiltration by α -SMA
267 positive fibroblasts (Figure 1A, D and E).

268 *2. Heterozygous deficiency of PHD2 is not protective against DSS induced*
269 *intestinal fibrosis*

270 PHD2 is the main regulator of HIF- α stability (2, 7, 68). PHD2 has been
271 previously reported to be involved in the regulation of the wound healing
272 response(32, 78, 80). To investigate whether the antifibrotic effects of DMOG
273 in intestinal fibrosis were mediated by regulation of the canonical PHD2/HIF
274 pathway, we compared the development of fibrosis in WT vs PHD2 $^{+/-}$ mice
275 exposed to DSS and allowed to recover for 8 days. Figure 2A shows
276 representative images of mouse colon cross-sections stained with H&E, picro-
277 sirius red or immunofluorescence for α -SMA, comparing WT and PHD2 $^{+/-}$
278 mice. DSS caused colitis in WT mice (Figure 2A, B and D). Furthermore,
279 intestinal inflammation was accompanied by profound fibrosis with formation
280 of α -SMA fibroblast aggregates (Figure 2A and 2E) and increased mucosal and
281 submucosal deposition of collagen (Figure 2A and 2F). As demonstrated
282 previously, WT mice treated with DMOG had significantly reduced DAI scores
283 (Figure 2B) and reduced severity of fibrosis with reduced fibroblast infiltration
284 and mucosal collagen deposition (Figure 2A, 2E and 2F). In contrast to DMOG

285 treated mice, PHD2^{+/-} mice were fully susceptible to DSS induced colitis with
286 severe signs of disease reflected by high DAI scores (Figure 2C), severe
287 inflammation (Figure 2A and 2D) and significant fibrosis (Figure 2A, 2E and
288 2F). These results show that reduced activity of PHD2 alone does not
289 ameliorate intestinal fibrosis, suggesting that the antifibrotic actions of DMOG
290 are likely independent of the inhibition of PHD2 and canonical activation of the
291 HIF pathway.

292 *3. Hydroxylase inhibition directly inhibits TGF- β 1 induced fibroblast*
293 *activation.*

294 In order to investigate whether the effects of hydroxylase inhibition on fibrosis
295 were due to its anti-inflammatory actions or to direct anti-fibrotic effects on
296 fibroblasts, we investigated the effects of hydroxylase inhibitors on TGF- β 1-
297 induced fibroblast activation. As shown in Figure 3A, human colonic CCD-
298 18Co fibroblasts stimulated with 1ng/ml of TGF- β 1 for 48 hours underwent a
299 phenotypic transformation into myofibroblasts, characterised by increased
300 expression of α -SMA stress fibres. Moreover, western blot analysis revealed a
301 TGF- β 1 mediated induction of both α -SMA and collagen-1(α) expression
302 (Figure 3B). Treatment with either DMOG or JNJ1935 prior to TGF- β 1
303 stimulation reduced differentiation of the cells into myofibroblasts as shown by
304 reduced presence of α -SMA stress fibres (Figure 3C). Moreover, a reduction of
305 both α -SMA and collagen-1(α)-1 was observed in cells treated with the

306 hydroxylase inhibitors prior to TGF- β 1 stimulation (Figure 3D-F). Taken
307 together, these data suggest that the antifibrotic actions of hydroxylase
308 inhibitors may be due to their ability to block TGF- β mediated fibroblast
309 activation. Moreover the fact that JNJ1935, which evokes significantly lower
310 activation of HIF than DMOG, caused an equivalent reduction of fibrotic
311 markers to DMOG further suggests that the effects of DMOG are HIF
312 independent(4).

313 *4. Hydroxylase inhibition does not affect activation of the TGF- β -Smad*
314 *signalling pathway*

315 To investigate how hydroxylase inhibition suppresses the effects of TGF- β 1, we
316 analysed the effects of hydroxylase inhibitors on activation of the canonical
317 TGF-Smad signalling pathway. To facilitate transfection studies, mouse
318 embryonic fibroblasts (MEF) were used in these experiments. DMOG reduced
319 the number of α -SMA positive MEF that migrated into a wound space under the
320 influence of TGF- β 1 in a wound healing assay, compared to cells that were
321 stimulated with TGF- β 1 in the absence of hydroxylase inhibition (Figure 4A).
322 This effect correlated with the ability of DMOG to significantly reduce TGF- β 1
323 induced expression of α -SMA mRNA, confirming that hydroxylase inhibitors
324 exert similar effects on TGF signalling in MEF and indicating that such effects
325 are also reflected at the transcriptional level (Figure 4B). We next tested the
326 effect of DMOG on Smad signalling. Stimulation with TGF- β 1 induced

327 phosphorylation of Smad2 and Smad3 at 30 to 120 minutes (Figure 4C-4E).
328 Treatment with DMOG failed to reduce Smad2 phosphorylation at any time
329 point, although it modestly reduced levels of pSmad3 at 2 hours (figure 4C-4E).
330 The combination of hydroxylase and proteasome inhibition did not further
331 affect the phosphorylation of Smad2/3, confirming that DMOG does not
332 enhance pSmad degradation (Figure 4C-4E). To further analyse the effects of
333 hydroxylase inhibitors on Smad pathway functionality, MEF were transfected
334 with the Smad luciferase reporter SBE4-Luc. This construct incorporates four
335 copies of the Smad binding element (SBE) in its promoter. Co-transfection of
336 MEF with SBE4 and Smad2 or Smad3 overexpression plasmids showed
337 activation of the reporter as expected (Figure 4F), with Smad3 more active than
338 Smad2 in this regards in the absence of TGF- β 1 (Figure 4F). Similarly, TGF- β 1
339 activated the SBE4-Luc reporter in a time-dependent fashion that was maximal
340 at 8 hours (Figure 4G). However, DMOG did not reverse the effect of TGF- β 1
341 on Smad-dependent luciferase activity (Figure 4G). Together these results show
342 that the effects of hydroxylase inhibitors on responses to TGF- β 1 are not due to
343 blockade of the canonical Smad signalling pathway.

344 *5. Hydroxylase inhibitors reduce TGF- β 1 dependent activation of the non-*
345 *canonical extracellular regulated kinase (ERK) signalling pathway*

346 To investigate an alternative explanation for the effects of the hydroxylase
347 inhibitors on TGF- β 1 signalling, we next examined the effects on non-canonical

348 signalling. Amongst MAPK pathways implicated in TGF- β 1 responses, c-Jun
349 N-terminal kinases (JNK) and ERK have been described for their activation in
350 fibrotic disease and involvement in the expression of fibrotic markers(3, 26, 31,
351 38, 42, 43, 55, 72). In the present study, areas of the mucosa of both WT and
352 PHD-2 \pm mice where α -SMA positive fibroblasts are concentrated exhibited
353 increased pERK nuclear staining, confirming the activation of the ERK pathway
354 in fibrotic colons (Figure 5A). In vitro, DMOG did not affect TGF- β 1 induced
355 phosphorylation of JNK (data not shown). In contrast, DMOG reduced TGF- β 1-
356 induced phosphorylation of ERK (pERK) at 8 hours post stimulation (Figure 5B
357 and 5D). Interestingly, DMOG did not affect the phosphorylation of the
358 upstream kinase MEK at the same time point, indicating that the kinase cascade
359 was specifically affected at the level of ERK activation (Figure 5C and 5E).
360 Phosphorylation of the linker region of Smad2, which is mediated directly by
361 ERK(11, 29, 69), was also moderately decreased by DMOG further confirming
362 the effects of DMOG on phosphorylation of ERK (Figure 5B). The hydroxylase
363 inhibitor JNJ1935 and atmospheric hypoxia were also examined for their effects
364 on ERK activation. JNJ1935 caused a time-dependent reduction in TGF- β 1-
365 induced ERK phosphorylation that was already present at 6 hours and was
366 profound at 8 hours post TGF stimulation (Figure 5F and 5G). Of note, hypoxia
367 did not significantly affect the ability of TGF- β 1 to activate this kinase cascade
368 (Figure 5F and 5G). In order to further analyse the mechanism of hydroxylase
369 inhibitor mediated reduction of ERK phosphorylation, we compared the effect

370 of DMOG and JNJ1935 on ERK and MEK phosphorylation to that of the MEK
371 inhibitor PD184161 (Figure 5H). While DMOG and JNJ1935 reduced ERK
372 phosphorylation without affecting that of MEK, the reduction of ERK
373 phosphorylation caused by PD184161 was accompanied by a dramatic increase
374 in phosphorylated MEK (Figure 5H). Taken together, these results show that
375 hydroxylase inhibitors modulate TGF-ERK signalling, which is a potential
376 mechanism whereby they exert anti-fibrotic effects.

377 *6. The antifibrotic effect of hydroxylase inhibitors is independent of HIF*

378 Our previous in vivo studies demonstrated no protective effects of PHD2
379 deficiency against fibrosis, thus providing evidence that the antifibrotic effects
380 of DMOG are HIF independent. To further demonstrate this hypothesis, we
381 compared the response of WT and PHD2^{+/-} MEF to TGF- β 1. Heterozygous
382 deficiency of PHD2 did not block ERK phosphorylation in response to TGF- β 1,
383 nor did it prevent the ability of DMOG or JNJ1935 to reduce levels of pERK
384 (Figure 6A). Moreover, stimulation of PHD2^{+/-} cells with TGF- β 1 caused an
385 increase in production of α -SMA and CTGF mRNA (Figure 6B and 6C). The
386 increment was significantly higher than that found in WT cells, but was
387 nevertheless abrogated when PHD2^{+/-} cells were treated with DMOG (Figure
388 6B and 6C). Next, we analysed whether reducing HIF in hydroxylase inhibitor
389 treated cells would affect the ability of the compounds to reduce the expression
390 of fibrotic markers. Drugs such as Digoxin and Camptothecin have been

391 described as potent HIF inhibitors (8, 46, 76). As has been previously described,
392 we found treatment with either digoxin or camptothecin to reduce DMOG
393 mediated HIF-1 α protein stabilization in MEF (data not shown). However, this
394 reduction of HIF did not affect the ability of DMOG to reduce the expression of
395 Acta2 (α -SMA) or Ctgf RNA (Figure 6D and 6E). Together these data suggest
396 that the antifibrotic effects of hydroxylase inhibitors are independent of HIF.

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408 **Discussion**

409 Hydroxylase inhibitors have recently been shown in clinical trials for anemia to
410 be well tolerated by patients and therefore represent a potentially important new
411 class of therapeutic agents(9, 28). We and others have previously demonstrated
412 protective effects of these reagents in multiple models of murine colitis.
413 Therefore, the potential for repurposing hydroxylase inhibitors for the treatment
414 of colitis is a realistic possibility. Here, we investigated whether the beneficial
415 effects of hydroxylase inhibition in intestinal inflammation extend to
416 inflammation-associated fibrosis, using the DSS-induced murine colitis model.
417 We show that treatment with the pan-hydroxylase inhibitor DMOG during the
418 post-DSS recovery period reduces intestinal fibrosis in addition to its known
419 anti-inflammatory effects. We also show that hydroxylase inhibitors are able to
420 block TGF- β 1 mediated activation of intestinal fibroblasts as well as MEF, and
421 that they do so at least in part via a HIF-independent manner. At the molecular
422 level, the inhibitors reduce TGF- β 1 mediated activation of the ERK signalling
423 pathway, thus providing mechanistic insight into the anti-fibrotic actions of
424 these compounds.

425 The DSS model of colitis has been used to investigate intestinal fibrosis by
426 allowing long recovery periods after exposure to DSS(63, 71). Another
427 approach to the DSS colitis model often used to investigate fibrosis is the so
428 called multicycle DSS model, where the animals are exposed to multiple cycles

429 of DSS followed by one week recovery periods. In a study comparing the
430 different approaches, Ding et al showed the presence of fibrosis already after
431 the first DSS cycle of DSS and highlighted that C57BL6 mice do not show
432 mucosal healing after DSS and progress towards chronic colitis(17). Moreover,
433 the notion that fibrosis is also a common complication in ulcerative colitis
434 further validates the potential for the DSS recovery model of colitis as a model
435 for the study of intestinal fibrosis(23). As previously described, our results show
436 that mice allowed to recover after exposure to DSS have evident signs of
437 inflammation and fibrosis even 8 or 14 days after DSS exposure. We also noted
438 that there are differences in disease progression between the different strains of
439 mice used. C57BL6 were susceptible to a lower dose of DSS and rapidly
440 developed colitis showing signs of disease already during the DSS exposure
441 period. In contrast, Swiss 129 mice did not show signs of disease initially, even
442 if exposed to a higher dose of DSS. However, this mice developed clear signs of
443 colitis after the DSS period and progressed towards more severe colitis and
444 fibrosis. In our studies, treatment of mice with DMOG during the post-DSS
445 recovery period reduced fibrosis as shown by the reduced presence of mucosal
446 α -SMA positive fibroblasts and reduced sub-mucosal collagen deposition.
447 These effects were associated with reduced inflammation, showing that
448 hydroxylase inhibitors might prove beneficial if used as treatments in
449 established colitis. Of note, a number of recent studies have indicated that the
450 inhibition of PHD2 positively regulates healing responses, supporting the

451 hypothesis that hydroxylases might be novel targets for the modulation of
452 wound healing and therefore, fibrosis(32, 78, 80). In our studies, however,
453 PHD2^{+/-} mice were not protected against chronic inflammation or fibrosis
454 induced by DSS. Furthermore, PHD2^{+/-} MEF did not demonstrate reduced
455 sensitivity to TGF- β 1 stimulation compared to WT, suggesting that the
456 beneficial effects of hydroxylase inhibitors in wild-type mice are likely
457 independent of the PHD2/HIF axis. This HIF independent mechanism is also
458 supported by the antifibrotic effects obtained with JNJ1935, a drug used at a
459 concentration that does not inhibit FIH and thus, does not strongly activate HIF.
460 We further tested the involvement of HIF in hydroxylase inhibitor anti-fibrotic
461 actions by inhibiting HIF stabilization in DMOG treated cells. As previously
462 demonstrated, digoxin and camptothecin reduced HIF-1 α in DMOG treated
463 cells(8, 46, 76). However, this did not affect the ability of DMOG to reduce the
464 expression of α -SMA and CTGF in TGF- β 1 stimulated cells. This provided
465 further evidence that the anti-fibrotic action of hydroxylase inhibitors is HIF
466 independent.

467 Fibrosis results from overactive wound healing and is mainly attributed to the
468 profibrogenic factor TGF- β 1(16, 42, 49, 67). In our studies, hydroxylase
469 inhibitors demonstrated specific anti-fibrotic actions by targeting the ability of
470 TGF- β 1 to up-regulate the production of various key fibrosis markers in
471 cultures of human colonic fibroblasts and MEF. However, DMOG failed to

472 block the phosphorylation of Smad2/3 or the activation of a Smad luciferase
473 reporter in response to TGF- β 1. Rather, hydroxylase inhibitors reduced TGF- β 1
474 mediated activation of the ERK signalling pathway, which has been shown to
475 play important roles in fibrosis(31, 42, 55) and which we found to be active in
476 areas of intestinal fibrosis. Thus, we propose that hydroxylase inhibitors target
477 the activation of the non-canonical TGF-ERK signaling pathway to cause the
478 inactivation of the ERK mediated fibrotic response (Figure 7). Together, these
479 results indicate that hydroxylase inhibitors, in addition to their previously
480 described anti-inflammatory effects, have beneficial effects against intestinal
481 fibrosis at least in part through inhibition of the TGF-ERK signaling pathway.
482 Of note, the inhibitory effects of hydroxylase inhibitors on ERK
483 phosphorylation were not seen for its upstream kinase MEK. Indeed,
484 comparison between hydroxylase inhibitors and a MEK inhibitor suggested a
485 different mechanism for the inhibition of ERK phosphorylation between the two
486 types of drugs. While MEK inhibitors reduced ERK phosphorylation but
487 induced a higher level of phosphoMEK, hydroxylase inhibitors reduced
488 phosphoERK but did not affect phosphoMEK. In order to gain further insights
489 to the possible mechanism of this PHD inhibitor mediated regulation of ERK
490 phosphorylation, we hypothesised that PHD inhibitors could upregulate dual
491 specificity phosphatases (DUSP). DUSP are known to inactivate MAPK and
492 have been shown to be regulated by hypoxia in multiple studies(5, 35, 41, 54,
493 56, 66). Hydroxylase inhibition did not increase the expression of DUSPs

494 investigated (data not shown). Furthermore, our results are in line with recently
495 published reports showing beneficial effects of hydroxylase inhibitors in
496 fibrosis(34, 57, 62, 73, 77). Of note, previous studies have described deleterious
497 effects for HIF activation in fibrotic pathologies(27, 70). The current study is
498 not in contradiction with this possibility, as we show that the intestinal
499 antifibrotic actions of hydroxylase inhibitors are HIF-independent.

500 The ability of hydroxylase inhibitors to regulate the stability of HIF- α subunits
501 may have not only beneficial effects, but also potential adverse effects. A
502 widely discussed side effect of hydroxylase inhibitors is the activation of HIF-
503 dependent EPO production(6, 30, 33), which is seen as an important limitation
504 to the use of these drugs. However, this ability to increase EPO is currently
505 being investigated as a therapy for anemia(6, 9, 28). In IBD, anemia is one of
506 the most common extraintestinal symptoms and it is estimated that one third of
507 IBD patients suffer from anemia(21). Moreover, anemia is reportedly predictive
508 of severe and disabling progression in IBD(39). Therefore, the activation of
509 EPO production with hydroxylase inhibition could be a secondary beneficial
510 effect in IBD patients where fibrosis and anemia are often co-incidental
511 outcomes. On the other hand, the use of targeted release forms that allow
512 specific local delivery of hydroxylase inhibitors to the colon could minimize
513 systemic exposure. In recent work, we demonstrated that the use of DMOG

514 mini-spheres formulated to target delivery to the colon achieved anti-
515 inflammatory effects while minimizing systemic side effects(65).

516 IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is increasing
517 in prevalence, especially in developed countries(45, 51). Advanced stages of
518 IBD are often associated with excessive wound healing causing fibrosis and
519 tissue scarring. While much IBD research seeks to unravel the early causes of
520 pathology, less attention has been paid to long-term complications. Despite the
521 advances achieved in the treatment of IBD with novel therapies such as anti-
522 TNF- α antibodies that help to maintain remission(14, 44), IBD is still in need of
523 improved therapeutic approaches. Intestinal fibrosis is a major indication for
524 surgery in IBD as 75-80% of CD patients are estimated to require surgical
525 intervention due to the formation of fibrotic scars leading to intestinal
526 obstructions(47, 59, 63). Further, the importance of fibrosis in a number of other
527 chronic inflammatory diseases such as chronic kidney disease or interstitial
528 pulmonary fibrosis(16, 18, 24, 59, 74) and the lack of effective means to limit
529 progression towards fibrosis suggest that treatments which target fibrotic
530 disease may represent an important therapeutic advance. We have shown that
531 hydroxylase inhibitors have promising anti-fibrotic effects, potentially due to
532 their actions on the non-canonical TGF-ERK signalling pathway.

533

534

535 **Grants**

536 This work was supported by Science Foundation Ireland (SFI: 11/PI/1005) and
537 Deutsche Forschungsgemeinschaft.

538 **Disclosures**

539 Mario C Manresa: nothing to disclose; Murtaza M Tambuwala: nothing to
540 disclose; Praveen K Rhadakrishnan: nothing to disclose; Jonathan Harnoss:
541 nothing to disclose; Eric Brown: nothing to disclose; Miguel S Cavadas:
542 nothing to disclose; Ciara E Keogh: nothing to disclose; Alex Cheong: nothing
543 to disclose; Kim E Barrett: nothing to disclose; Eoin P Cummins: nothing to
544 disclose; Martin Schneider: nothing to disclose; Cormac T Taylor: nothing to
545 disclose

546 **Acknowledgements**

547 The authors thank the Conway Institute imaging and genomics CORE services
548 for the excellent technical assistance.

549 **Author contributions**

550 Mario Manresa, Murtaza Tambuwala, Jonathan Harnoss, Martin Schneider and
551 Cormac Taylor participated in the design of this study. Mario Manresa, Murtaza
552 Tambuwala, Praveen Radhakrishnan, Eric Brown, Miguel Cavadas, Ciara
553 Keogh and Alex Cheong participated in the acquisition of data. Mario Manresa,

554 Murtaza Tambuwala, Eoin Cummins, Kim Barrett and Cormac Taylor
555 participated in the analysis and interpretation of the data. Mario Manresa, Kim
556 Barrett and Cormac Taylor participated in the drafting of this manuscript.

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837 **Tables**

838

Target	Supplier	Code
α-SMA	Abcam	Ab7817
Collagen-1(α)-1	Santa Cruz Biotechnology	sc-8784
HIF-1α	BD Transduction Laboratories	#610958
HIF-1α (clone H1a67)	Merck Millipore	MAB5382
pSmad2 (carboxyterminal)	Merk Millipore	AB3849
pSmad2 (linker)	Cell Signalling Technology	#3104
pSmad3	Cell Signalling Technology	#9520
TotalSmad2	Cell Signalling Technology	#3103
TotalSmad3	Cell Signalling Technology	#9513
pERK	Santa Cruz Biotechnology	sc-7383
TotalERK	Cell Signalling Technology	#9102
Alexa fluor 488 (anti-mouse)	Thermo Fisher Scientific	A-21202

839 **Table 1. Antibodies list.** List of antibodies used for immunostaining and
840 western blot protein analysis.

Target	Sequence	Chemistry	Source
m-α-SMA (Acta2)	5'-TGCTGTCCCTCTATGCCTCT-3', sense		
	5'-GCAGGGCATAGCCCTCATAG-3', antisense	Sybr green	Self-designed
Ctgf	Commercially available Taqman probe	Taqman	Applied Biosystems
Eukaryotic 18S rRNA	Commercially available Taqman probe	Taqman	Applied Biosystems

841 **Table 2. Primer list.** List of target genes analysed and the complementary primers used for
842 qRT-PCR. The list includes the source of the primers.

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849 **Figure legends**

850 **Figure 1.** *DMOG reduces fibrosis in DSS induced colitis.* A, representative images of mouse colon
851 stained with hematoxylin and eosin (H&E), Picrosirius red imaged with double polarized light
852 (collagen) and immunofluorescence histochemistry (α -SMA). White arrows point areas of major
853 collagen accumulation or α -SMA positive infiltrates. Quantification of disease activity index (DAI)
854 (B), assessment of tissue inflammation (C), submucosal collagen deposition (D) and α -SMA positive
855 infiltration (E). n=4, * $p\leq 0.05$; ** $p\leq 0.01$; *** $p\leq 0.001$.

856 **Figure 2.** *Heterozygous deficiency of PHD2 does not protect against intestinal fibrosis in DSS-*
857 *induced colitis.* A, representative images of mouse colon from WT and PHD2 \pm mice stained with
858 H&E, Picrosirius red imaged with transmitted light (collagen) and immunofluorescence histochemistry
859 (α -SMA). Quantification of disease activity index (DAI) in WT mice comparing natural recovery to
860 IP DMOG treatment (B); Quantification of DAI comparing natural recovery between WT and
861 PHD2 \pm mice (C); Assessment of tissue inflammation (D); Quantification of α -SMA positive
862 staining (E); Quantification of percentage of mucosal collagen (F). n ≥ 3 , * $p\leq 0.05$; ** $p\leq 0.01$; ***
863 $p\leq 0.001$.

864 **Figure 3.** *Hydroxylase inhibition reduces TGF- β 1 induced human colonic fibroblast*
865 *activation.* A, representative images of CCD-18Co cells stimulated with 1ng/ml TGF- β 1 and
866 stained for α -SMA using immunofluorescence histochemistry with DAPI as a nuclear
867 counter-stain (n=2). B, representative western blot of α -SMA and collagen-1(α)-1 in CCD-
868 18Co cells stimulated with 1ng/ml TGF- β 1 (n=3); C, representative images of CCD-18Co
869 stimulated with 1ng/ml TGF- β 1 and treated with 1mM DMOG or 100 μ M JNJ1935, stained
870 for α -SMA using immunofluorescence histochemistry with DAPI as a nuclear counter-stain
871 (n=3). D, representative western blot of α -SMA and collagen-1(α)-1 in CCD-18Co cells
872 treated with 1mM DMOG or 100 μ M JNJ1935 and stimulated with 1ng/ml of TGF- β 1 for 48
873 hours (n=10). E, densitometry of α -SMA in CCD18Co treated as described in D (n=10). F,
874 densitometry of collagen-1 α in CCD18Co cells treated as described in D (n=10).

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876 **Figure 4.** *Hydroxylase inhibition does not affect TGF- β 1 induced Smad activation.* A,
877 representative images of MEF monolayers, treated with 1mM DMOG for 1 hour, wounded
878 and then stimulated for 24 hours with 10ng/ml TGF- β 1, stained for α -SMA using
879 immunofluorescence histochemistry with DAPI as a nuclear counter-stain (n=4). B, qRT-
880 PCR of α -SMA in MEF stimulated with 1ng/ml TGF- β 1 and treated with 1mM DMOG
881 (n=3); C, representative western blot of pSmad2, pSmad3, TotalSmad2, TotalSmad3, HIF-1 α
882 and β -Actin in MEF stimulated with 1ng/ml TGF- β 1 for 1/2h or 1h or 2h and treated with
883 1mM DMOG for 1h +/- 1/2h 10nM MG132 pre-treatment (n=6); D, densitometry of pSmad2
884 (n=6); E, densitometry of pSmad 3 (n=6). F, luciferase production in MEF transfected with
885 SBE4-Luc and co-transfected with pCMV5B-Smad3-flag or pCMV5-Smad2-HA (n=4); G,
886 luciferase production in MEF transfected with SBE4-Luc and stimulated with 1ng/ml TGF-
887 β 1 and treated with 1mM DMOG (n=4). * p \leq 0.05; ** p \leq 0.01; *** p \leq 0.001.

888 **Figure 5.** *Hydroxylase inhibition reduces TGF- β 1 induced ERK activation.* A, representative images
889 of mouse colon cross-sections stained for α -SMA using immunofluorescence histochemistry and
890 stained for phospho-ERK (pERK) using immunohistochemistry (n \geq 3). Red arrows point areas of
891 abundant pERK positive cells, which are often coincident to areas where activated fibroblasts
892 accumulate. B, representative western blot of pERK, TotalERK, pSmad2 linker, TotalSmad2 and β -
893 Actin in MEF stimulated with 1ng/ml TGF- β 1 and treated with 1mM DMOG (n=6); C,
894 representative western blot of pMEK and β -Actin in MEF stimulated with 1ng/ml TGF- β 1 and
895 treated with 1mM DMOG (n=5). D, densitometry for pERK of the western blots described in B
896 (n=6); E, densitometry for pMEK of the western blots described in C (n=5). F, representative
897 western blot of pERK, TotalERK, pMEK, TotalMEK and β -Actin in MEF stimulated with 1ng/ml
898 TGF- β 1 and treated with 100 μ M JNJ1935 or 1% oxygen (n \geq 3); G, densitometry for pERK of the
899 western blots described in F; H, representative western blot of pSmad2 linker, pERK, pMEK and β -
900 Actin in MEF treated with 1mM DMOG or 100 μ M JNJ1935 or 0.3mM PD184161 and stimulated
901 with 1ng/ml of TGF- β 1 (n=4). * p \leq 0.05; ** p \leq 0.01.

902 **Figure 6.** *The anti-fibrotic effect of hydroxylase inhibitors is HIF independent.* A, representative
903 western blot of pERK, TotalERK and β -Actin in WT vs PHD2^{+/-} MEF stimulated with 1ng/ml TGF-
904 β 1 and treated with 1mM DMOG or 100 μ M JNJ1935 (n=4); qRT-PCR of α -SMA (B) and CTGF (C)
905 in WT vs PHD2^{+/-} MEF stimulated with 1ng/ml TGF- β 1 and treated with 1mM DMOG (n \geq 3).
906 Quantitative real time PCR of α -SMA (D) or CTGF (E) in MEF pre-treated with 100 μ M digoxin or
907 2 μ M camptothecin for 30 minutes, then with 1mM DMOG for 1 hour and then stimulated with
908 1ng/ml TGF- β 1 for 8 hours (n \geq 3). * p \leq 0.05; ** p \leq 0.01.

909 **Figure 7.** *Purposed mechanism for hydroxylase inhibitor mediated anti-fibrotic actions.* Upper panel
910 displays the combination of TGF- β 1 activated Smad dependent (canonical) and non-canonical (ERK
911 dependent) signaling pathways. Lower panel shows our purposed model for hydroxylase inhibition
912 antifibrotic action. Pharmacologic inhibition of oxygen sensing hydroxylases mediates the long term
913 dephosphorylation of the non-canonical TGF-ERK signaling pathway and reduces the crosstalk
914 between ERK and Smad pathways. This causes a reduction of the TGF mediated fibroblast activation
915 and ameliorates fibrosis in a mouse model of colitis.

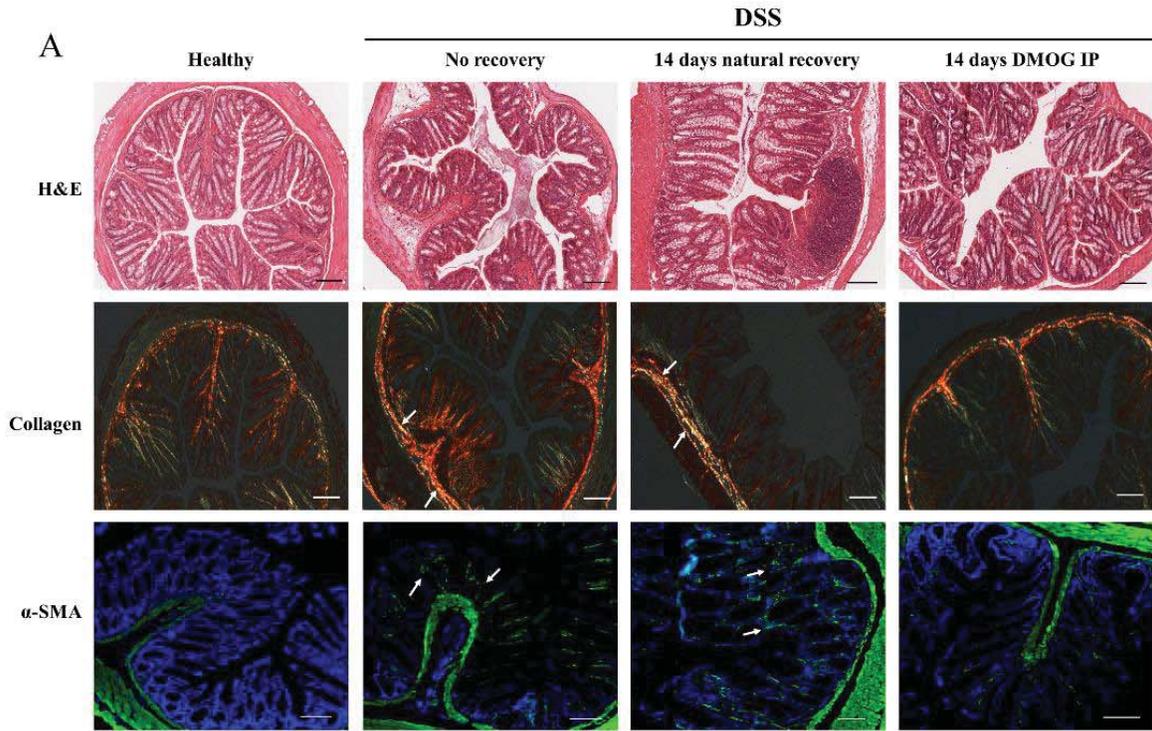
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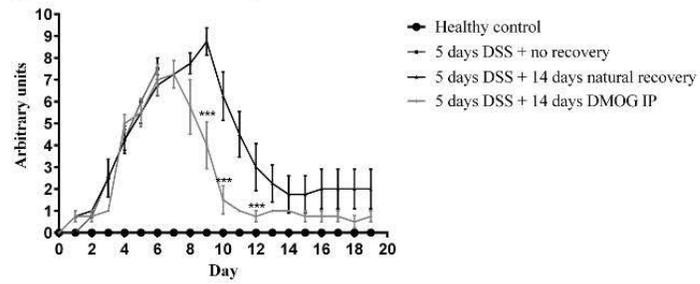
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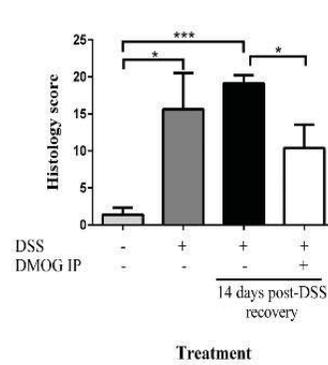
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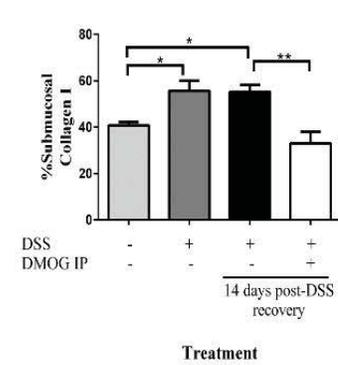
B Disease activity index



C Assessment of tissue inflammation



D Submucosal Collagen I



E α-SMA positive staining (mucosa)

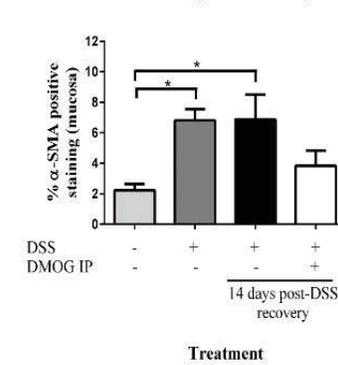


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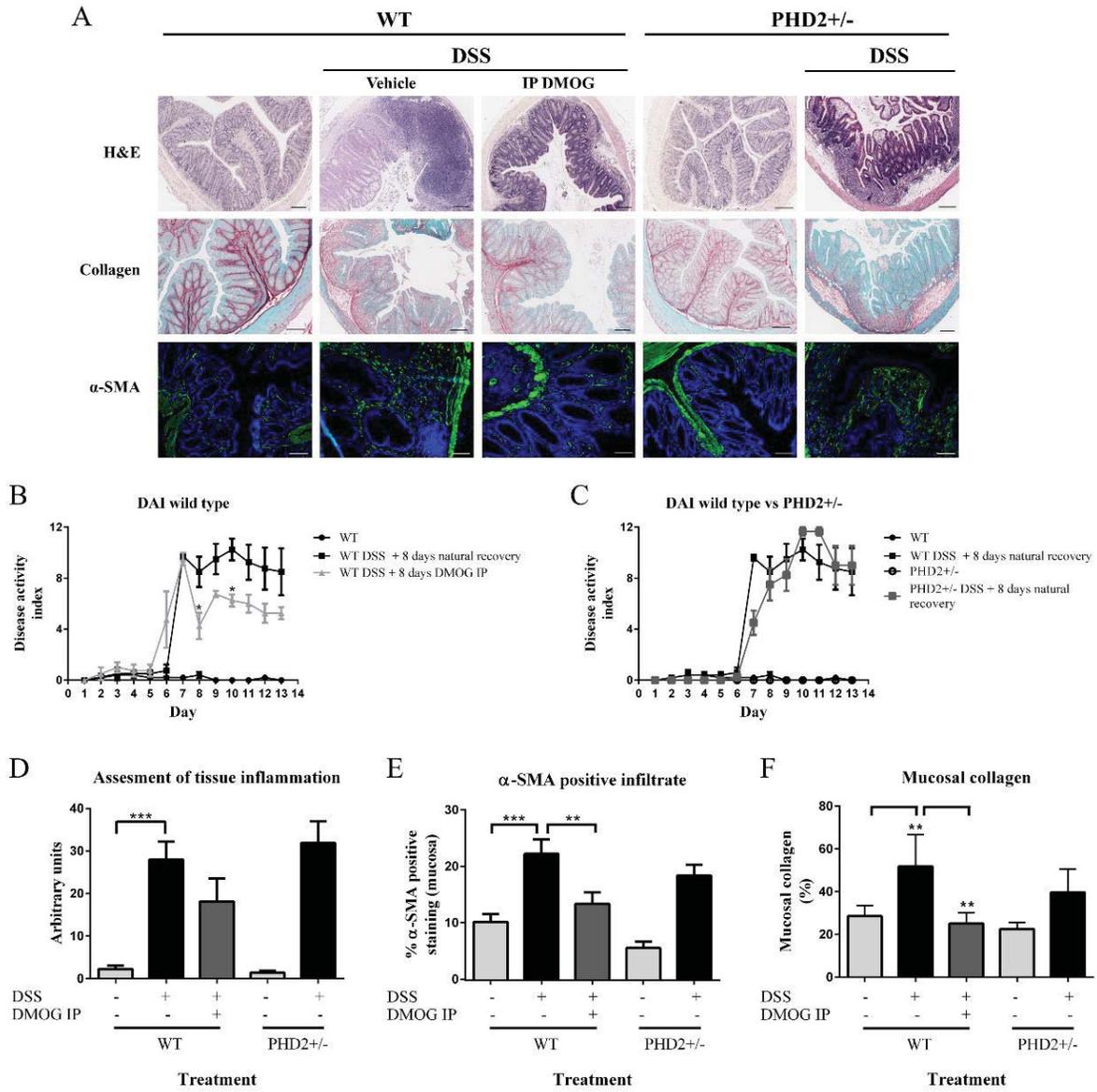
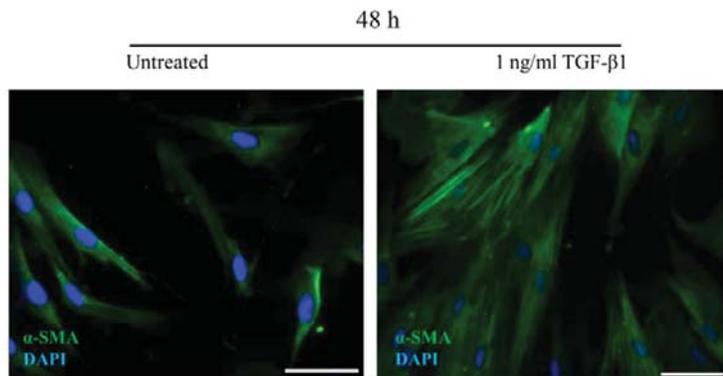
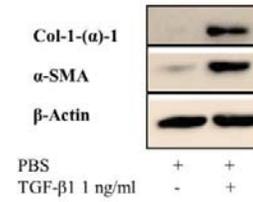


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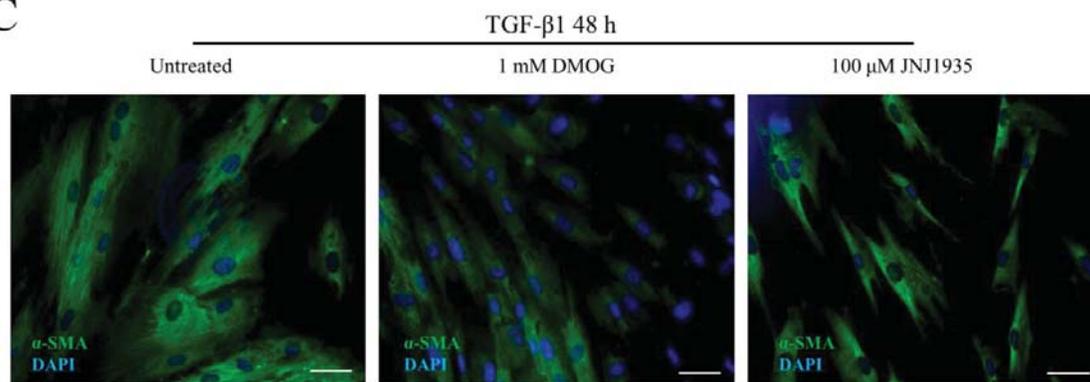
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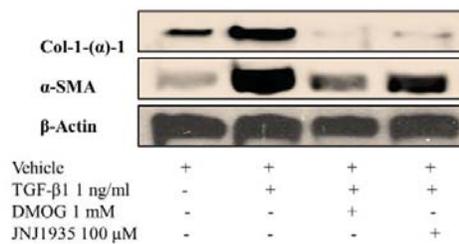
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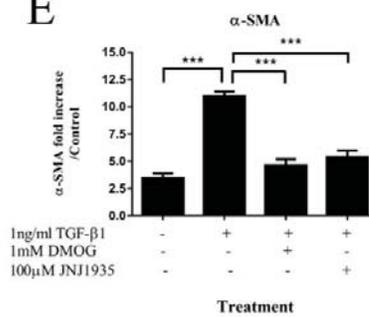
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D



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F

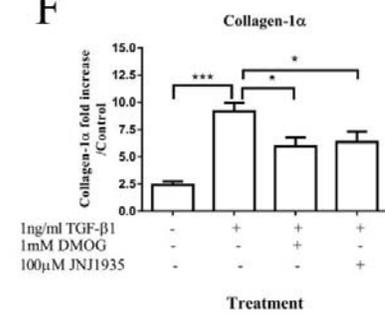


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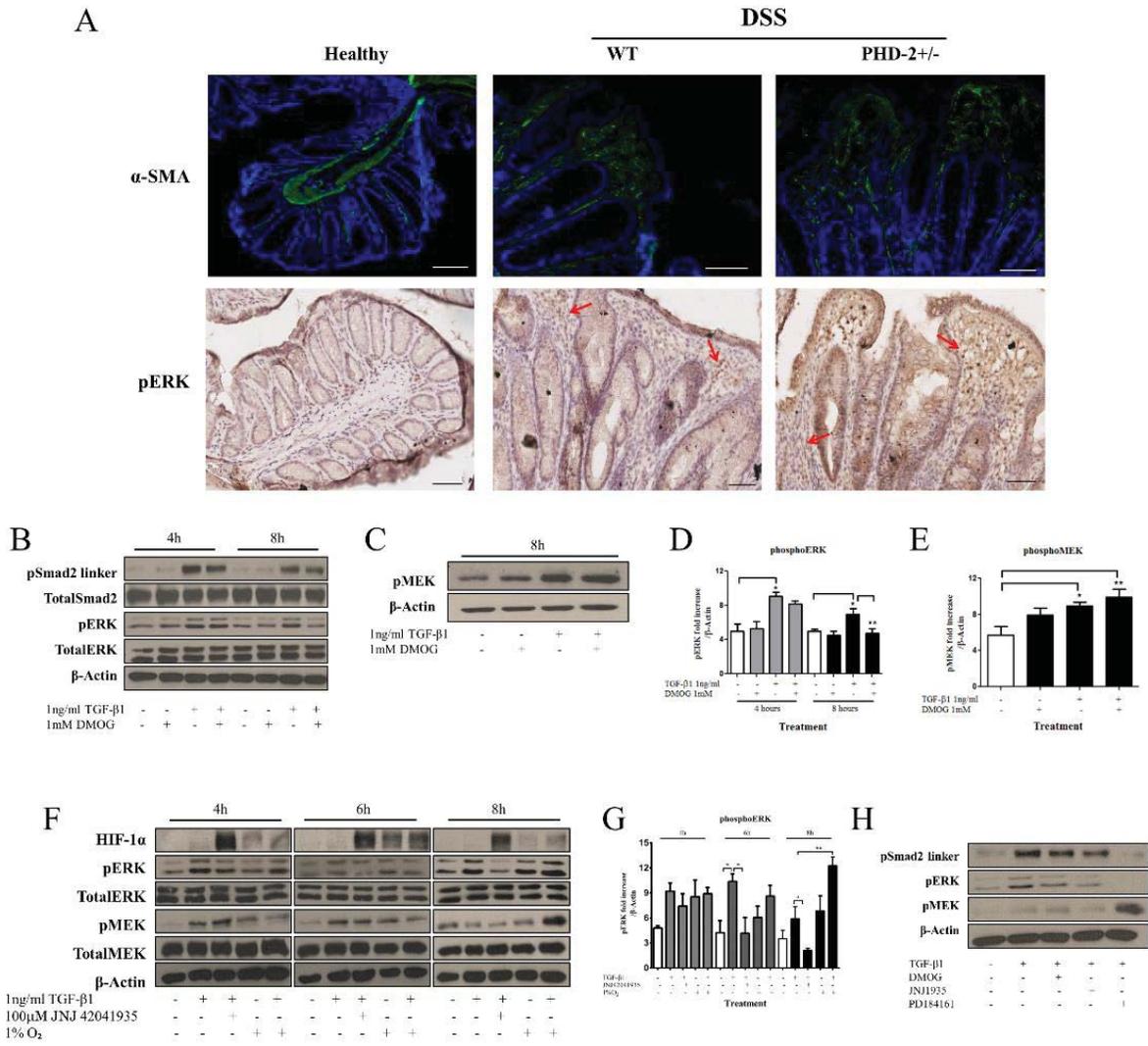


Figure 6

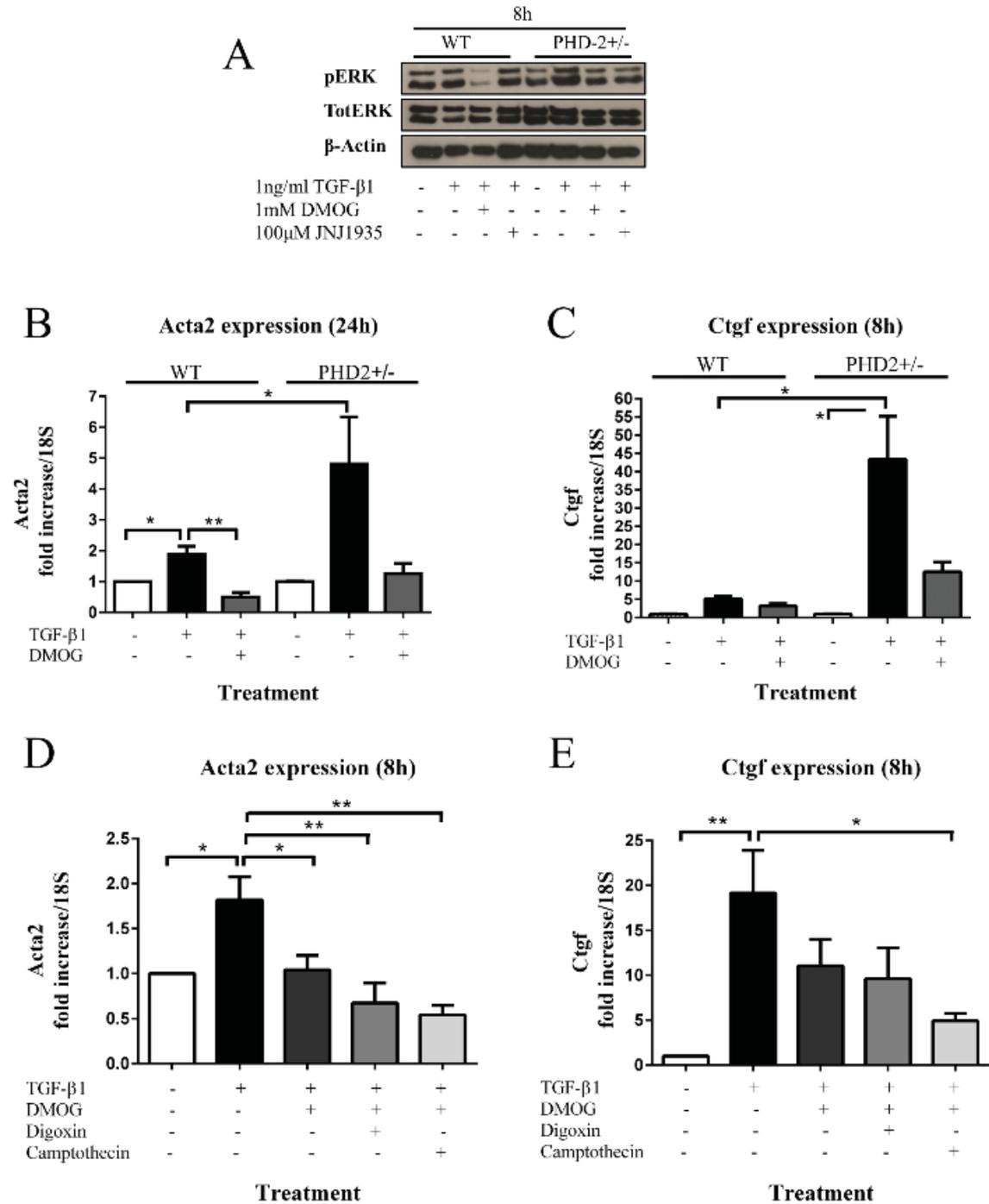


Figure 7

