Supplemental Material for

The HIF-prolyl hydroxylases have distinct and non-redundant roles in colitis-associated cancer

Kilian B. Kennel¹, Julius Burmeister¹, Praveen Radhakrishnan¹, Nathalia A. Giese¹, Thomas Giese², Martin Salfenmoser¹, Jasper M. Gebhardt¹, Moritz J. Strowitzki¹, Cormac T. Taylor³, Ben Wielockx⁴, Martin Schneider¹, and Jonathan M. Harnoss¹,*

¹Department of General, Visceral and Transplantation Surgery, University Hospital Heidelberg, Heidelberg, Germany
²Institute of Immunology, University Hospital Heidelberg, Heidelberg, Germany
³School of Medicine, Systems Biology Ireland and the Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland
⁴Institute for Clinical Chemistry and Laboratory Medicine, Dresden University of Technology, Dresden, Germany

*Corresponding author: Department of General, Visceral and Transplantation Surgery, Heidelberg University, Im Neuenheimer Feld 420, 69120 Heidelberg. Phone: +49-6221-566110; Email: Jonathan.harnoss@med.uni-heidelberg.de

Authorship note: KBK and JB are co-first authors and contributed equally to the manuscript. MSch and JMH are co-last authors and contributed equally to the manuscript.

This file includes:

- Supplemental Methods
- Supplemental Figures 1-7 including Figure legends
- Supplemental Tables 1 and 2
- References for Supplemental Data
SUPPLEMENTAL METHODS

Histology, immunohistochemistry, immunofluorescence

For histology and IHC, tumor-bearing colons were fixed as “swiss rolls” using formalin and ethanol, embedded in paraffin, and cut at 5 µm thickness (1). A subset of tumors was frozen and processed for cryosectioning and subsequent IF staining. Paraffin-embedded tissue sections were dewaxed using xylene and a graded series of ethanol. H&E staining was performed for microscopic analysis of mucosal damage using a previously described scoring system (2) (Supplemental Table 1). Two blinded observers assessed histology scores using a Zeiss Axiostar Plus microscope in combination with an Axiocam MRC camera (Zeiss, Jena, Germany). For IHC, citrate-based antigen retrieval was performed (Dako Target Retrieval Solution, Agilent, Santa Clara, California, USA., #S1699) and sections were incubated overnight at 4°C with the following primary antibodies: PCNA (1:500, Abcam, Cambridge, UK, #265585), CC3 (1:100, Cell Signaling Technology (CST), Danvers, Massachusetts, USA, #9661), F4/80 (1:100, BioRad, Hercules, California, USA, #MCA497), CD3 (1:100, Abcam, #5690), pSTAT3 (1:100, CST, #9145), ERK1/2 (1:1000, CST, #9102), pERK1/2 (1:1000, CST, #9101). For IF on cryosections, a CD11c primary antibody was used (1:100, ThermoFisher Scientific, Waltham, Massachusetts, USA, #14-0114-82). After incubation with appropriate horse radish peroxidase or FITC (for IF-coupled secondary antibodies), detection was carried out using the Liquid DAB+ Substrate Chromogen System (Dako, #K3468). Quantification of positive cells was performed by two blinded investigators using a Zeiss Axiostar Plus microscope in combination with an Axiocam MRC camera (Zeiss).

qRT-PCR

RNA was isolated from murine colonic tumor and mucosa samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany, #74104). cDNA was synthesized using the ImProm-II™ Reverse Transcription System (Promega, Mannheim, Germany, #A3800). qRT-PCR was performed on a LightCycler 480 system (Roche, Mannheim, Germany) using SYBR Green as a dye. Relative transcript expression was analyzed employing the △△Ct method with 18S rRNA (Rn18s) or actin beta (Actb) as housekeeping genes. Primer sequences are listed in Supplemental Table 2.
Immunoblot
Protein from size- and location-matched AOM/DSS tumors was isolated using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, #80004). Isolated protein was quantified using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, #23225), and equal amounts were loaded for gel electrophoresis. Primary antibodies against STAT3 (1:1000, CST, #9139), pSTAT3 (1:1000, CST, #9145), ERK1/2 (1:1000, CST, #9102), pERK1/2 (1:1000, CST, #9101), ACTB (1:5000, Abcam, #8227) as well as an HRP-conjugated secondary antibody (1:5000, Abcam, #6721) were used for detection of the respective proteins.

Flow cytometry
The following antibodies were used to characterize lymphoid populations in AOM/DSS tumors: APC-R700-CD45 (30-F11) (BioLegend, 103128), Brilliant Violet 605-CD3 (17A2) (BioLegend, 100237), PE-CF594-CD4 (RM4-5) (BD, 562285), APC-C7, CD8a (53-6.7) (BioLegend, 100714), BB515-CD19 (1D3) (BD, 564531), Brilliant Violet 421-CD25 (PC61) (BD, 562606), APC-CD127, (SB/199), (BD, 564175), CD335/NKp46 (29A1.4) (BD, 560757).
For characterization of myeloid populations in AOM/DSS tumors, the following antibodies were used: APC-R700-CD45 (30-F11) (BioLegend, 103128), PE-Cy7-CD11b (M1/70) (BioLegend, 101216), Brilliant Violet 421-CD11c, (N418) (BioLegend, 117330), AF488-CD80 (16-10A1) (BioLegend, 104716), Brilliant Violet-786-CD86 (GL1) (BioLegend, 105043), PE-CF594-CD163 (S15049I) (BioLegend, 155316) PE -CD197/CCR7 (4B12) (BD, 560682), AF647-CD206 (MR5D3) (BD, 565250), APC-C7-F4/80 (BM8) (BioLegend, 123118), PerCP-Cy5.5-Ly-6C (HK1.4) (BioLegend, 128012) Brilliant Violet 605-Ly-6G (1A8) (BioLegend, 127639), BV510-MHC class II (M5/114.15.2) (BioLegend, 107636), BUV395-CD3 (17A2), (BD, 740268), BUV395-CD19 (1D3) (BD, 563557), BUV395-CD335/NKp46 (29A1.4) (BD, 740326). Incubation was performed in Brilliant Stain Buffer (BD, 563794). DAPI was added before data acquisition to identify viable cells.
**Isolation of BMDMs**

BMDMs were isolated as previously described (3). Briefly, femora and tibiae of WT and Phd2^+/− mice were flushed with PBS, and bone marrow cells were differentiated to macrophages in RPMI-1640 medium supplied with 10% FCS, 1% Penicillin/Streptomycin, 2 mM L-Glutamine and 10 ng/ml murine M-CSF (R&D Systems, Minneapolis, Minnesota, USA, #416-ML) for seven days prior to experiments. For experiments, BMDMs were incubated for 24 hours with control media (RPMI-1640 medium supplied with 1% FCS), 100 ng/ml LPS in control media (Sigma-Aldrich, St. Louis, Missouri, USA, #L2630), 20 ng/ml TNFα in control media (R&D Systems, #410-MT), or 20 ng/ml IL-4 in control media (R&D Systems, #404-ML).
Supplemental Figure 1. (A) Disease activity index (DAI) scores from WT (n = 10), Phd1−/− (n = 6), Phd2−/− (n = 5), and Phd3−/− (n = 9) mice over the course of AOM/DSS treatment. The DAI was calculated every other day. (B) qRT-PCR analysis of pro-inflammatory mRNA expression in non-tumorous colon tissue samples from WT (n = 9), Phd1−/− (n = 9), Phd2−/− (n = 11), and Phd3−/− (n = 11) mice at day 84. Statistical significance was calculated using 2-way ANOVA (A) or 1-way ANOVA with Dunnett’s multiple comparisons test (B). *P < 0.05.
Supplemental Figure 2. (A) Macroscopic quantification of AOM/DSS-induced tumors. Pooled number of tumors per mouse (left: WT: n = 27, Phd1−/−: n = 21, Phd2+/−: n = 20, Phd3−/−: n = 20 mice) and pooled size of individual tumors (right: WT: n = 220, Phd1−/−: n = 63, Phd2+/−: n = 255, Phd3−/−: n = 243 tumors) of 4 studies in total. Statistical significance was calculated using 1-way ANOVA with Dunnett’s multiple comparisons test. *P < 0.05, **P < 0.01, ****P < 0.0001.
Supplemental Figure 3. (A) Quantification of epithelial nuclear β-catenin immunostaining in WT (n = 28) and Phd2\(^{−/−}\) (n = 54) tumors and representative histological images. Scale bar = 100 μm. (B) Quantification of epithelial nuclear pSTAT3Y705 immunostaining in WT (n = 19) and Phd1\(^{−/−}\) (n = 12) tumors and representative histological images. Scale bar = 100 μm. (C, D) qRT-PCR analysis of Egfr (C) and EGFR ligands mRNA expression (D) in WT (n = 16) and Phd2\(^{−/−}\) (n = 16) tumors. (E) Re-analysis of a publicly available high-density microarray data set that includes transcriptomes from size- and location-matched AOM/DSS-induced and sporadic ApomMin\(^{+}\) tumors and respective controls (4). Statistical significance was calculated using 1-way ANOVA with Dunnett’s multiple comparisons test (A) or Student’s t test (B - E). \(^{*}\)P < 0.05.
Supplemental Figure 4. (A) Flowcytometry gating strategy for myeloid populations in AOM/DSS tumors as previously established (5). First, single live cells were identified based on their FSC and SSC properties and DAPI staining. Next, immune cells (CD45+) were gated, and T, B, and NK cells excluded via CD3, CD19, and CD335 dump staining. Myeloid cells were then defined as neutrophils (CD45+, CD3-, CD19-, CD335-, Ly-6G+, CD11b+), Tumor-associated macrophages (TAMs, CD45+, CD3-, CD19-, CD335-, Ly-6G-, F4/80+, CD11b low), resident macrophages (res Macs, CD45+, CD3-, CD19-, CD335-, Ly-6G-, F4/80+, CD11b high), dendritic cells (DCs, CD45+, CD3-, CD19-, CD335-, Ly-6G-, F4/80-, CD11b+, MHCIIm, CD11c+), monocytes (Mono, CD45+, CD3-, CD19-, CD335-, Ly-6G-, F4/80+, CD11b+, MHCIIm, CD11c-, Ly-6C+). Representative contour plots from a Phd2−/− mouse.
Supplemental Figure 5. (A) Flow cytometry gating strategy for lymphoid populations in AOM/DSS tumors. First, single live immune cells were identified based on their FSC and SSC properties, CD45, and DAPI staining. Next, lymphoid cells were defined as B cells (CD45+, CD19+), NK cells (CD45+, CD3-, CD335+), NKT cells (CD45+, CD3+, CD335+), T cells (CD45+, CD3+), Th cells (CD45+, CD3+, CD4+), regulatory T cells (Tregs, CD45+, CD3+, CD4+, CD127-, CD25+) and cytotoxic T cells (CD45+, CD3+, CD8+). Representative contour plots from a WT mouse. (B) Median fluorescence intensity for M1 (CD80, CD86, CCR7) and M2 (CD163, CD206) macrophage polarization markers within the TAM population in tumors from Phd2+/− (n = 6) and WT (n = 6) control mice. Statistical significance was calculated using Student's t test.
Supplemental Figure 6. (A) Quantification of epithelial nuclear β-catenin immunostaining in Phd2^{f/f} (control, n = 27) and Vav:Cre-Phd2^{f/f} mice (n = 36) and representative histological images (right). Scale bar = 100 µm. (B-C) qRT-PCR analysis of Egfr (B) and EGFR ligands (C) mRNA expression in control (n = 14) and Vav:Cre-Phd2^{f/f} mice (n = 14).
Supplemental Figure 7. (A) Macroscopic quantification of AOM/DSS-induced tumors in Phd2<sup>ff</sup> (control) and Villin:Cre-Phd2<sup>ff</sup> mice. Number of tumors per mouse (left; control: n = 8, Villin:Cre-Phd2<sup>ff</sup>: n = 8 mice) and size of individual tumors (right; control: n = 80, Villin:Cre-Phd2<sup>ff</sup>: n = 64 tumors). Representative macroscopic images of colons from control and Villin:Cre-Phd2<sup>ff</sup> mice. Arrows indicate colitis-associated tumors. Scale bar = 2 mm. (B) H&E stainings of colons from control and Villin:Cre-Phd2<sup>ff</sup> mice. Scale bar = 2 mm. (C) Quantification of epithelial PCNA immunostaining in control (n = 17) and Villin:Cre-Phd2<sup>ff</sup> (n = 15) tumors and representative histological images (bottom). Scale bar = 25 μm. (D) Quantification of epithelial CC3 immunostaining in control (n = 16) and Villin:Cre-Phd2<sup>ff</sup> (n = 16) tumors and representative histological images (bottom). Scale bar = 25 μm. (E) Quantification of epithelial nuclear pSTAT3Y705 immunostaining in control (n = 20) and Villin:Cre-Phd2<sup>ff</sup> (n = 16) tumors and representative histological images (bottom). Scale bar = 25 μm. (F-G) qRT-PCR analysis of EGFR ligand Ereg (F) and Il6 and Il11 (G) mRNA expression in control (n = 6) and Villin:Cre-Phd2<sup>ff</sup> (n = 6) tumors. Statistical significance was calculated using Student’s t test.
**Supplemental Tables**

| Score | Colonic epithelial damage                           | Inflammatory cell infiltration | Score |
|-------|-----------------------------------------------------|--------------------------------|-------|
| 0     | Normal epithelium                                   | Mucosa Normal                  | 0     |
| 1     | Hyperproliferation, irregular crypts, goblet cell loss | Mucosa Mild                    | 1     |
| 2     | Mild to moderate crypt loss (10-50%)                | Mucosa Modest                  | 2     |
| 3     | Severe crypt loss (50-90%)                          | Mucosa Severe                  | 3     |
| 4     | Complete crypt loss, surface epithelium intact      | Submucosa Normal               | 0     |
| 5     | Small- to medium sized-ulcer (<10 crypt widths)     | Submucosa Mild to modest       | 1     |
| 6     | Large ulcer (≥ 10 crypt widths)                     | Submucosa Severe               | 2     |
|       |                                                     | Muscle/serosa Normal           | 0     |
|       |                                                     | Muscle/serosa Moderate to severe| 1     |

**Supplemental Table 1. Histological scoring criteria for DSS-induced colitis.** As previously described by Katakura et al. (2). Scores for epithelial damage and inflammatory cell infiltration (separately assessed for mucosa, submucosa, and muscle/serosa) are added, resulting in a minimum score of 0 and a maximum score of 12.
Supplemental Table 2. Primer sequences used for qRT-PCR

| Gene   | Primer type (FW, forward/REV, reverse) | Primer sequence   |
|--------|----------------------------------------|-------------------|
| II6    | FW                                     | TTCCTCTCTGCAAGAGACTTC |
|        | REV                                    | CTGTTGGGAGTGGTATCCTCTG |
| II11   | FW                                     | GGGGACATGAACCTGTGTTTGT |
|        | REV                                    | CAGGAGGGATCAGGTTAGGA |
| Mpo    | FW                                     | CTGCAAAACAGACAGACCC |
|        | REV                                    | AGCCATTTGCAAGTGGCA |
| Ptgs2  | FW                                     | TCCCATGGGTTGAGGAAAA |
|        | REV                                    | ACCCAGCTCTCTGCTTATGA |
| Cxcl1  | FW                                     | ACCCAAAACGAATCAGACC |
|        | REV                                    | TGTCAGAAGCCAGTACC |
| Cxcl2  | FW                                     | GCCAGGCAATCAGTACC |
|        | REV                                    | CTTCCTTACCCGCTAACG |
| Myc    | FW                                     | GAACCAGAGACACAGGAT |
|        | REV                                    | GTTGGTCTCTCTTGCTTATGA |
| Birc5  | FW                                     | TGCCTGGAAGGCTAGAACAA |
|        | REV                                    | ACAAAGTGCTCCAGCC |
| Bcl2l1 | FW                                     | GCCAGGCAATCAGTACC |
|        | REV                                    | CTTCCTTACCCGCTAACG |
| Ereg   | FW                                     | GACATGGACGGCTACTGCTT |
|        | REV                                    | TGTCAGAAGCCAGTACC |
| Rn18s  | FW                                     | ACAAAGTGCTCCAGCC |
|        | REV                                    | CCATCCAACTGGTAGAGCC |
| Actb   | FW                                     | TATAAACCCGCGCCGC |
|        | REV                                    | TCAATCGGAGTAGGCTTAT |
| Areg   | FW                                     | CAGGAGGCAATCAGGTTA |
|        | REV                                    | AAAACCTGGACTGGCTT |
| Egf    | FW                                     | TTCTGGGTTCAGGACTG |
|        | REV                                    | GAACAAACTCTGGCCCTT |
| Hbegf  | FW                                     | AGGACCTTGGAGGAGCAA |
|        | REV                                    | CCAATTCCTTCTTCTGCTT |
| Btc    | FW                                     | ATGAGCCACCCAGCGGCTGAGT |
|        | REV                                    | TAAACGTTAACATATGGCTGT |
| Epgn   | FW                                     | GAGCAGAGAGAGAGAGGCT |
|        | REV                                    | GTCTTCCAGAAAGATGAGAG |
| Tgfa   | FW                                     | AGGCGGAGAGAAGCCCATC |
|        | REV                                    | TCACTTCTGCTGGGTTAGCAA |
| Egfr   | FW                                     | GAAAGTGCCCGAAAAT |
|        | REV                                    | TCGTAGTAGTCAGGGCCA |


REFERENCES

1. Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, et al. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell.* 2004;118(3):285-96.

2. Katakura K, Lee J, Rachmilewitz D, Li G, Eckmann L, and Raz E. Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. *J Clin Invest.* 2005;115(3):695-702.

3. Kiss J, Mollenhauer M, Walmsley SR, Kirchberg J, Radhakrishnan P, Niemietz T, et al. Loss of the oxygen sensor PHD3 enhances the innate immune response to abdominal sepsis. *J Immunol.* 2012;189(4):1955-65.

4. Neufert C, Becker C, Tureci O, Waldner MJ, Backert I, Floh K, et al. Tumor fibroblast-derived epiregulin promotes growth of colitis-associated neoplasms through ERK. *J Clin Invest.* 2013;123(4):1428-43.

5. Olesch C, Sirait-Fischer E, Berkefeld M, Fink AF, Susen RM, Ritter B, et al. S1PR4 ablation reduces tumor growth and improves chemotherapy via CD8+ T cell expansion. *J Clin Invest.* 2020;130(10):5461-76.