

1 **Deep Visual Proteomics advances human colon organoid
2 models by revealing a switch to an *in vivo*-like phenotype upon
3 xenotransplantation**

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21 **Abstract**

22 **Intestinal epithelial damage predisposes to chronic disorders like inflammatory bowel
23 disease. The organoid model allows cultivation, expansion and analysis of primary
24 intestinal epithelial cells and has been instrumental in studying epithelial behavior in
25 homeostasis and disease. Recent advances in organoid transplantation allow studying
26 human epithelial cell behavior within the intestinal tissue context. However, it remained
27 unclear how organoid transplantation into the colon affects epithelial phenotypes, which is
28 key to assessing the model's suitability to study human epithelial cells. We employed Deep
29 Visual Proteomics, integrating AI-guided cell classification, laser microdissection, and an
30 improved proteomics pipeline to study the human colon. This created an in-depth cell type-
31 resolved proteomics resource of human intestinal epithelial cells within human tissue, *in*
32 *vitro* organoids, and the murine colon post-xenotransplantation. Our findings reveal that *in*
33 *vitro* conditions induce a proliferative organoid phenotype, which was reversible upon
34 transplantation and adjustment of organoid culturing conditions.**

35

36

37 **Introduction**

38 The intestinal epithelium forms an integral barrier between the intestinal lumen, filled with
39 microbiota and dietary components, and the lamina propria containing immune cells and
40 fibroblasts. Continuous proliferation of epithelial stem cells located within the epithelial crypts
41 ensures constant replenishment of intestinal epithelial cells (IECs). As stem cell progeny move
42 towards the crypt top, they cease to divide and differentiate terminally, establishing a
43 heterogeneous continuum along the crypt axis. These terminally differentiated IECs include
44 absorptive colonocytes, mucus producing goblet cells, and hormone secreting enteroendocrine
45 cells, which all perform key functions in intestinal physiology¹.

46 Epithelial maintenance is key for human health and requires tight molecular regulation
47 balancing cell proliferation, differentiation and death. Murine models have provided substantial
48 mechanistic insights into these intricate relations. There are, however, clear differences between
49 the human and mouse, e.g. unique cell types identified in the human intestine², highlighting the
50 need for human models. Addressing mechanistic questions in humans *in vivo* is challenging, and
51 organoids^{3–5} have emerged as an important model system to culture primary human cells and
52 allow experimental manipulation. Human intestinal organoids have provided insights into e.g. cell
53 fate choices, with applications in molecular medicine, drug testing and cellular therapies^{1,6–9}.
54 Conventional organoid culture features epithelial cells, but lacks other cell types present in the
55 intestinal mucosa, such as immune cells and fibroblasts¹. To address this limitation, orthotopic
56 transplantation models have recently been developed^{10,11}. They enable the transplantation of wild-
57 type or genetically engineered mouse or human organoids into the murine colon to mechanistically
58 dissect epithelial phenotypes within the mucosal microenvironment, which was previously only
59 possible in mouse models. Furthermore, autologous transplantation of organoids into patients with
60 impaired IEC phenotypes has great therapeutic potential in regenerative medicine, e.g. for
61 inflammatory bowel disease (IBD) and short bowel syndrome^{6,8}. This tractable xenotransplantation
62 system enables the assessment of human IEC phenotypes in the mucosal microenvironment^{11,12},
63 but we still only have limited knowledge on how well human IECs transplanted into the murine
64 colon recapitulate human IECs *in vivo*.

65 Fully leveraging the potential of human organoids requires in-depth characterization and
66 validation of organoid models^{1,13}, which necessitates an accurate reference data set of their *in vivo*
67 counterpart. Such a resource could guide future evaluation of disease-related changes, cellular
68 and disease markers, and improvement of *in vitro* model systems. An accurate assessment of
69 cellular phenotypes should account for their spatial context, especially in delicately organized
70 tissues like the colon mucosa. Spatial transcriptomics and fluorescent *in situ* hybridization (FISH)-
71 based techniques have provided valuable insights into the cellular heterogeneity of the colon^{14,15}.
72 These approaches, however, require pre-defined target panels and are biased by current
73 knowledge. Single cell RNA-sequencing (scRNASeq), facilitates in-depth characterization of
74 cellular phenotypes^{16–18}, but lacks spatial information. Typically, it also requires cellular dissociation

75 and long enrichment protocols, which in itself can impact epithelial phenotypes¹⁹. In the context of
76 organoids, scRNASeq has been used to assess cellular composition^{5,20}, but in-depth phenotypic
77 benchmarking including direct comparison to the *in vivo* counterparts remains limited, especially
78 for the human colon.

79 Recent studies suggest that deep and sensitive proteomics provides more robust readouts for
80 cellular states than transcriptomes, while directly pinpointing functional consequences of
81 perturbation-induced changes^{21,22}. The sensitivity of proteomics has advanced massively in the
82 last decades from the quantification of a few thousand proteins from milligrams of input material in
83 the beginning of the millennium to comparable numbers from single cells to date^{22–24}. However, so
84 far none of these methods have reached substantially complete coverage of cell type-specific
85 proteomes. To address this, we here substantially further develop our Deep Visual Proteomics
86 (DVP)²⁵ pipeline, which employs high-resolution fluorescence imaging, AI-guided cell
87 segmentation and classification, single-cell isolation by laser capture microdissection, and high-
88 sensitivity proteomics. To date, the conventional DVP pipeline generally yielded up to 5,000
89 proteins by combining a few hundred contours of single cell contours of the same type²⁵. Our
90 improved workflow using low flow gradients and the novel Orbitrap Astral analyzer²⁶, improved
91 proteome coverage substantially, from even fewer contours. This allowed us to build a spatial
92 proteome atlas of the human colon mucosa with unprecedented cell type-specific proteome depth.
93 Importantly, the increased depth of protein quantifications at decreased input amounts enabled us
94 to robustly and accurately benchmark human colon organoids grown *in vitro* and transplanted into
95 the murine colon.

96 Our findings reveal that despite a robust correlation between *in vitro* and *in vivo* proteomes,
97 IECs grown as organoids *in vitro* display high proliferation and low functional signatures. Strikingly,
98 this is reverted upon xenotransplantation, rendering xenotransplanted human IECs a valuable tool
99 to dissect human IEC phenotypes and illustrating that organoids retain their ability to reform colonic
100 epithelium. Combined with iterative, proteomics guided improvements in organoid cell culture
101 conditions this is a promising approach in regenerative medicine.

102

103 **Results**

104

105 **DVP enables in-depth spatial proteomic profiling of cellular populations in the human** 106 **colon**

107 The assessment of human organoid models requires the determination of the *status quo* of the
108 human colon mucosa. We made use of DVP (Fig. 1A) to generate a high sensitivity spatial
109 proteome atlas of the human colon mucosa and analyze organoid models. In total, we analyzed
110 11 human colon tissue sections, 15 sections of organoids *in vitro*, and 50 sections of transplanted
111 organoids.

112 The analysis of the human colon mucosa included different populations of colonic epithelial
113 cells (EPCAM⁺) and their microenvironment (lamina propria fibroblasts (PDGFRA⁺), immune cells
114 (CD45⁺)) (Fig. 1B). Intestinal stem cells can be identified by *LGR5* expression²⁷, but it has proven
115 difficult to generate antibodies for reliable detection of LGR5. Alternative strategies for isolating
116 human intestinal stem cells have been developed based on expression of EPHB2^{28,29}, PTK7³⁰ and
117 OLFM4³¹, however, it remained challenging to detect epithelial stem cells in the human colon
118 mucosa. We capitalized on the DVP technology to address this pertinent problem, enabling us to
119 separate the epithelial crypt bottoms (enriched for stem cells, hereafter referred to as “crypt
120 bottom”) from the upper part of the crypt (hereafter referred to as “upper crypt”) (Fig. 1C) based on
121 spatial context. We used cellpose to segment high-resolution images for cell detection³². The
122 resulting cell shapes and marker staining intensity were used to classify epithelial, goblet, immune
123 cells and fibroblasts from the crypt bottom and upper crypt region using the biological image
124 analysis software (BIAS) resulting in contours (one contour ≈ one cell in a 5 µm tissue section)
125 (Fig. 1C). Technological limitations concerning availability of material and reliance on cellular
126 markers for in-depth analysis of specific cellular subpopulations have so far hindered the
127 characterization of functional states and phenotypes of human colonic epithelial cell
128 subpopulations at protein levels. To address this, we isolated ~500 contours per population by
129 laser capture microdissection, lysed the collected contours, digested the proteins and performed
130 proteome acquisition on the Evosep One liquid chromatography system coupled to an Orbitrap
131 Astral mass spectrometer (Experimental Methods). With this approach, we achieved
132 unprecedented sensitivity of cell populations directly isolated from fresh-frozen tissue, featuring
133 8,865 unique proteins across all cell populations and a median of 6,780 unique proteins per sample
134 with a throughput of 40 samples per day (Fig. 1D-E, S1A-B). The limited sample amount from
135 transplanted organoids restricted us to collecting a maximum of 100 contours from transplanted
136 stem cells and ~200 contours of transplanted epithelial cells in the upper crypt. Remarkably, the
137 quantification of these samples still yielded ~5,000 or ~7,000 proteins, respectively (Fig. S1A).

138 Downstream principal component analysis (PCA) of the resulting data revealed that the
139 samples from the human colon mucosa separated into two main clusters according to epithelium
140 and lamina propria (immune cells and fibroblasts) along PC1, and further distributed according to
141 the position along the crypt axis (bottom or top) along PC2 (Fig. 1F). To assess the reliability of
142 identification and isolation of the different cell populations, we next assessed the abundance of
143 previously described cellular markers for the isolated subpopulations in our sample set (Fig. 1C,
144 G, S1C) and identified high expression of keratin (KRT)20 in upper crypt epithelial cells, Ephrin-
145 type B receptor (EPHB)2 in crypt bottom epithelial cells, mucin (MUC)2 in goblet cells, thymocyte
146 antigen (THY)1 in fibroblasts, as well as cluster of differentiation (CD)3E and human leukocyte
147 antigen (HLA)-DRA in immune cells, thereby validating our human colonic mucosa proteome atlas.
148 Interestingly, within the epithelial and lamina propria clusters, sample location along the crypt axis
149 (upper crypt/bottom) rather than cell type drove their distribution (Fig. 1F). Differential activity of

150 WNT and BMP signaling along the crypt axis regulate cellular organization, proliferation and
151 differentiation within the intestinal epithelium, suggesting that these pathways might partially drive
152 observed differences. The protein transgelin (TAGLN) was associated with the crypt bottom
153 compartment irrespective of the cell type (Fig. S1D). In line with high WNT activity around the
154 epithelial stem cell niche in the crypt bottom, TAGLN⁺ stromal cells have been identified as WNT
155 producers³³. The protein Zinc Finger ZZ-Type And EF-Hand Domain Containing (ZZEF)1, on the
156 other hand, was enriched in the upper crypt compartment (Fig. S1D). ZZEF1 acts as a
157 transcriptional regulator in cooperation with Krueppel-like factor (KLF)6 and KLF9³⁴ which regulate
158 IEC proliferation³⁵ and absorption³⁶, and might be modulated by the intestinal microbiota³⁷,
159 indicating a potential involvement in the integration of environmental stimuli into epithelial
160 phenotypes. The interplay between luminal inputs and intrinsic regulation of mucosal gradients
161 along the crypt axis and their molecular basis warrants further investigation.

162 In summary, we successfully generated a proteome atlas of the human colon mucosa in
163 unprecedented depth with our DVP approach, which reveals differentially regulated protein levels
164 along the crypt axis across cell types.

165

166 **DVP analysis reveals a robust correlation between human IECs *in vivo* and grown as 167 organoids**

168 For an in-depth characterization of human colon organoids at proteome level, we adapted the
169 DVP pipeline described above to organoids. The accurate and sensitive assessment of functional
170 cellular states at proteome level within a spatial context in combination with the *in vivo* proteome
171 atlas as reference data set enables the benchmarking of model systems for human IECs (Fig. 1).
172 Here we made use of a genetically engineered human colon cell organoid line, expressing the
173 fluorescent reporter TdTomato under the control of the LGR5 promoter¹² to identify epithelial stem
174 cells (Fig. 2A). This allowed us to use the DVP workflow described above to identify, isolate and
175 analyze human colonic stem cells (LGR5-TdTomato⁺ cells, hereafter referred to as “stem cells”),
176 LGR5-TdTomato⁻ cells (hereafter referred to as “LGR5⁻ cells”), and goblet cells to generate a
177 proteome atlas of human IECs grown as organoids *in vitro*. It should be noted that the half-life of
178 the reporter protein might be longer than LGR5, thus TdTomato⁺ cells could contain a fraction of
179 cells which have recently exited the stem cell state (e.g. transit amplifying progenitors). In the PCA,
180 samples clustered according to different epithelial populations (Fig. 2B) with PC1 separating stem
181 cells from the remaining IECs and PC5 separating goblet cells from stem cells and LGR5⁻ IECs.
182 Expectedly, KRT20 was enriched in the LGR5⁻ cells (Fig. 2C), the stem cell marker EPHB2 in
183 LGR5⁺ stem cells and MUC2 in goblet cells (Fig. 2C).

184 A comparison of significantly changed proteins in stem versus LGR5⁻ cells measured *in vitro*,
185 and those measured *in vivo* in the crypt bottom versus upper crypt respectively, showed a robust
186 correlation between the lower and upper crypt compartments *in vivo* and *in vitro* (Pearson
187 coefficient 0.77, Fig. 2D), which is in a similar range to the correlation of transcriptomes of murine

188 small intestinal IECs *in vitro* and *in vivo*^{38,39}. Notably, ~70% (crypt bottom) or ~60% (upper crypt)
189 of significantly enriched proteins in the respective populations *in vivo* were shared with organoids
190 grown *in vitro* (Fig. 2E). Among these, we identified a number of described markers associated
191 with the analyzed populations, indicating that key aspects of crypt bottom and upper crypt epithelial
192 cells are preserved in *in vitro* culture. The higher number of proteins identified as differentially
193 abundant *in vitro* is likely due to more homogenous populations isolated from *in vitro* than *in vivo*
194 conditions (e.g., LGR5⁺ cells/crypt bottom), which allow for a more robust comparison.

195 To conclude, with our DVP approach we successfully benchmark human colon organoids to
196 IECs *in vivo*, revealing a robust preservation of key compartment-associated features in organoids
197 and highlighting their applicability as a model system for human colon IECs *in vitro*.

198

199 **Orthotopic transplantation reverts organoid phenotypes to an *in vivo*-like state**

200 The transplantation of human organoids into the murine colon emerges as a novel model to
201 dissect human IEC phenotypes and behavior within the mucosal environment^{10,11}, but our current
202 knowledge on how well human IECs transplanted into the murine colon recapitulate human IECs
203 *in vivo* is limited to the assessment of selected markers for epithelial subpopulations^{10,11}. To
204 address this, we transplanted the genetically engineered human reporter organoids (Fig. 2A) into
205 the murine colon (Fig. 3A). Consistent with previous reports, the cultured cells integrated into the
206 murine colon mucosa and recapitulated the organotypic crypt structure featuring LGR5-TdTomato⁺
207 cells at the crypt bottom (Fig. 3B)^{10–12}. For a comprehensive, unbiased assessment of epithelial
208 phenotypes upon transplantation, we performed DVP analysis on the transplanted cells, focusing
209 on stem (LGR5-TdTomato⁺, hereafter referred to as “stem cells”) and remaining cells (LGR5-
210 TdTomato⁻, hereafter referred to as “LGR5⁻ cells”). Transplant size varies between mice and
211 sometimes comprises only a few crypts. In protocols that require tissue dissociation (e.g. for
212 scRNAseq), it can be challenging to efficiently recover these relatively rare cells. Furthermore, they
213 often include lengthy enrichment steps such as cell sorting, which impacts IEC phenotypes¹⁹. For
214 our DVP approach instead, we localized the transplants during sectioning, which enabled us to
215 efficiently isolate transplanted IECs directly from their mucosal microenvironment. Strikingly, our
216 DVP analysis revealed that transplanted organoids clustered with the *in vivo* IECs rather than
217 organoid samples (Fig. 3C). This is particularly remarkable given that all organoid samples derive
218 from the same organoid line (i.e., the same donor), while the IECs *in vivo* derive from three different
219 donors, indicating that the phenotypic shift across conditions is stronger than interindividual
220 differences.

221 To gauge the biological magnitude of this shift, we included the lamina propria cells (fibroblasts,
222 immune cells) isolated from the colon mucosa *in vivo* as outlier groups into the PCA (Fig. S3A).
223 Surprisingly, despite the robust correlation between IECs *in vivo* and *in vitro* observed above, the
224 distance between IECs grown as organoids *in vitro* and *in vivo* was very similar to the distance
225 along PC1 between lamina propria cells and IECs *in vivo*, which are different cell types. A major

226 driver for this differential clustering were components of the mucosal immunoglobulin A (IgA) (Fig.
227 S3B), an important adaptive immune component of the mucosal barrier, which is secreted into the
228 intestinal mucosa by B cells and subsequently transported into the intestinal lumen by IECs⁴⁰. This
229 indicates that the mucosal microenvironment has a significant impact on cellular proteomes across
230 cell types, which should be considered when translating findings from organoid studies to *in vivo*
231 phenotypes.

232 Collectively, the DVP analysis of orthotopically transplanted human colon organoids into the
233 murine colon demonstrates that the cellular environment strongly impacts on IEC proteome
234 profiles, pushing organoid phenotypes towards their *in vivo* counterparts.

235

236 To assess the cellular features driving phenotypic differences between IECs *in vitro* and within
237 the mucosa, we performed a Kruskal-Wallis test across all epithelial samples. Hierarchical
238 clustering of significantly changed proteins confirmed a separation of IECs grown *in vitro* from
239 those isolated from the mucosa (*in vivo*, transplant) (Fig. 3D). Protein abundance patterns among
240 these samples yielded eight clusters. Pathway analysis for the proteins within each cluster (Fig.
241 3E) revealed that signatures high in transcription (Cluster 4), translation (Cluster 1, 3), and
242 proliferation (Cluster 5) characterized organoids cultured *in vitro* (partially shared with crypt bottom
243 *in vivo* & transplanted stem cells), whereas *in vivo* and transplanted IECs were characterized by
244 signatures associated with mucosal barrier function⁴¹ (e.g. complement activation, Cluster 8),
245 functional features of mature IECs (e.g. ion transport, secretion, Cluster 7), and oxidative
246 phosphorylation (Cluster 6). A direct comparison between IECs *in vivo* and *in vitro* confirmed these
247 observations (Fig. S3C-H). The increased proliferative features *in vitro* were also evident as a
248 specific enrichment of proteins involved in proliferation in IECs *in vitro*, which was decreased upon
249 transplantation to levels similar to *in vivo* (Fig. 3F)⁴². To identify markers associated with upper
250 crypt IEC phenotypes *in vivo*, we next assessed the PC loadings to identify proteins that drive the
251 separation between IECs *in vivo* and *in vitro* (Fig. S3B). Here, carbonic anhydrase (CA)1 and
252 MUC17 were amongst the highest scoring proteins. CA1 mediates ion transport, which is key for
253 the regulation of water absorption in the intestine⁴³. MUC17 is a membrane mucin forming the
254 glycocalyx, an important barrier against bacterial attachment to the mucosa, which is compromised
255 in IBD⁴⁴ (Fig. S3I). In summary, components of two aspects of functional IECs *in vivo*, ion transport
256 and barrier function, are underrepresented in IECs grown *in vitro* under the conditions tested here.

257 Altogether, our DVP approach revealed that the *in vitro* culturing conditions used here induce
258 a high proliferation, low functional profile of IECs *in vitro*, and that these characteristics are
259 reversible upon transplantation into the colon mucosa. This underscores the value of transplanted
260 organoids as a system for the molecular dissection of epithelial phenotypes in a more *in vivo*-like
261 setting, and highlights their applicability in regenerative medicine, e.g., for approaches to replenish
262 impaired epithelium.

263

264 **Integrated DVP analysis identifies a human stem cell signature**

265 The use of fluorescent reporters has enabled studies of intestinal epithelial stem cells in mice
266 *in vivo* and in genetically engineered human organoids *in vitro* but it has so far been difficult to
267 specifically isolate and analyze human stem cells *in vivo* due to the lack of antibody-stainable stem
268 cell markers. Our study design uniquely allowed the collective in-depth proteome analysis of LGR5-
269 TdTomato⁺ human stem cells *in vitro* and upon xenotransplantation in comparison to stem cell-
270 enriched human IECs *in vivo*. The comparisons across these datasets enabled us to identify a
271 shared protein profile enriched in stem cells *in vitro* and upon transplantation, and crypt bottom
272 cells *in vivo*, which were downregulated in upper crypt cells *in vivo*. This human stem cell proteome
273 signature includes 48 proteins (Fig. 3G) and as expected, contains a number of proteins associated
274 with cell proliferation. The assessment of the expression patterns of these proteins via the Human
275 Protein Atlas⁴⁵ confirmed their localization at the crypt bottom *in vivo* (Fig. S4A-B). Notably, while
276 all identified proteins localized within the stem cell niche, their abundance towards the crypt's upper
277 part varied (Fig. S4A-B). Based on this, we postulate that markers with a relatively confined
278 expression such as EPHB3, meiotic recombination 11 (MRE11) and minichromosome
279 maintenance complex component 2 (MCM2) could be suitable markers for a strongly stem cell-
280 enriched IEC population. In comparison to previously published markers for stem cell enrichment
281 in the human colon such as PTK7, EPHB2 and OLFM4^{28,30,31}, expression of these markers is
282 more restricted to the crypt bottom (Fig. S4B). EPHB3 is a receptor tyrosine kinase involved in
283 regulation of stem cell positioning along the crypt axis and regulates mitogenic activity in
284 cooperation with WNT^{29,46}. As an antibody-stainable surface protein, we expect it to be a valuable
285 marker for the enrichment of human stem cells, e.g. in cell sorting, which would address a major
286 technical gap. MRE11⁴⁷ and MCM2⁴⁸ regulate DNA double-strand break repair and DNA
287 replication, respectively. Other markers such as PCNA, MCM3, MCM4 likely include transit
288 amplifying populations as well, in line with their roles in cell division^{49–51}.

289 With this, our DVP approach has enabled the identification of EPHB3 as a potential novel
290 surface marker for strong enrichment of stem cells, together with MRE11 and MCM2 as additional,
291 antibody-stainable markers.

292

293 **WNT withdrawal induces upregulation of *in vivo* IEC markers**

294 The protocols for expansion of IECs as organoids have been optimized for growth at the
295 expense of differentiation. This is achieved via activation of the WNT pathway (supplementation of
296 signals activating the canonical WNT pathway – WNT surrogate and R-spondin1), which is active
297 in the crypt bottom compartment *in vivo*, and inhibition of BMP signaling (supplementation of
298 Noggin), which is active in the upper crypt compartment *in vivo*^{3,5,52}. We hypothesized that these
299 conditions could be drivers of the observed *in vitro* characteristics shaped by high proliferation and
300 lower functional features when compared to the *in vivo* and transplanted IECs. In line with this,

301 both stem cells and LGR5⁻ cells *in vitro* were enriched for active WNT signaling⁵³ when compared
302 to their *in vivo* counterparts (Fig. S5A-B).

303 To address the impact of WNT and BMP signaling on epithelial phenotypes, we cultured
304 organoids *in vitro* under conventional (+WNT, Noggin, RSPO (WNR)) or differentiation (-WNR +/-
305 BMP) conditions^{12,54}. We observed a clear shift in organoid proteome profiles upon withdrawal of
306 WNR while the addition of BMP only had a minor additional effect (Fig 4A). As hypothesized, WNR
307 withdrawal led to a decrease in WNT activation (Fig. S5C-D). It furthermore induced a
308 downregulation of stem cell- and proliferation-associated proteins such as SOX9, MKI67, MCM2
309 and PCNA (Fig. 4B-C). This was also evident at a more global level when we assessed expression
310 of proteins assigned to the proliferation signature⁴² and our stem cell signature identified above
311 (Fig. 4D). At the same time, WNR withdrawal coincided with an upregulation of markers of mature
312 IECs, such as KRT20, as well as CA1 and MUC17, which we identified in the analysis above as
313 strongly associated with IECs *in vivo* (Fig. 4E). Similarly, the oxidative phosphorylation signature,
314 which was enriched *in vivo* compared to organoids (Fig. S3F) was increased upon WNR
315 withdrawal, indicating that IEC metabolic function is in part driven by IEC maturation state (Fig.
316 4F). Importantly, immunostaining of MUC17 in organoids upon WNR withdrawal revealed
317 increased abundance of MUC17 at the apical surface, suggesting glycocalyx formation under
318 these conditions. (Fig. 4G). Altogether, this indicates, as suggested previously, that withdrawal of
319 WNR indeed drives organoids towards a more *in vivo*, upper crypt-like phenotype⁵⁵.

320

321 Discussion

322 We here employ DVP to generate an in-depth proteome atlas of the human colon mucosa,
323 which we use to benchmark human colon organoids grown *in vitro* and upon orthotopic
324 xenotransplantation. We originally developed DVP as a spatial proteomics technology that enabled
325 the acquisition of the proteome of about 10 samples per day, quantifying up to 5,000 proteins from
326 input material equivalent to 100 – 200 cells²⁵. In our improved workflow, which includes coupling
327 the Evosep One liquid chromatography system to the Orbitrap Astral analyzer, throughput is
328 increased to 40 samples per day. Remarkably, total proteome acquisition time for this in depth,
329 functional organoid study encompassing 136 samples was only 88 hours. Despite faster
330 acquisition, we increased the proteome depth to a total of 8,865 unique proteins. This setup also
331 enabled the quantification of ~5,000 proteins from as little as 100 transplanted stem cell contours,
332 corresponding to only 20 intact cells. The increased proteome depth was essential to enable
333 conducting this study since it enabled us to identify low abundant proteins such as SOX9 or LGR5
334 from cells dispersed over several slides.

335 Based on this improved DVP pipeline, the benchmarking of human colon organoids reveals a
336 robust correlation of IECs grown *in vitro* and *in vivo*. Nevertheless, IECs grown *in vitro* display high
337 proliferation and altered functional and metabolic signatures compared to *in vivo*, which has
338 important implications for the use of organoids as models to dissect epithelial phenotypes. We

339 show that these features are driven by organoid culture conditions and are largely reverted upon
340 organoid transplantation into the murine mucosa, as well as, in part, by altering organoid culturing
341 conditions (WNR withdrawal). Altogether, our study validates the applicability of orthotopically
342 xenotransplanted organoids as tools to mechanistically dissect human IEC phenotypes in an *in*
343 *vivo*-like setting and highlights their potential to accurately replenish the intestinal epithelium in a
344 regenerative medicine approaches.

345 Human organoid models are instrumental for assessing key biological questions in a human
346 context. The premise that the organoid model truly recapitulates *in vivo* phenotypes, and an
347 awareness of its limitations, is crucial for the translatability of *in vitro* results to *in vivo* applications.
348 A key gap currently limiting the exploitation of the full potential of human organoids in biomedical
349 research is the characterization and validation of organoids as accurate models for human
350 biology^{1,7,13}. An in-depth characterization of native IEC states within their *in vivo* environment is
351 essential to establish a reference for benchmarking of human-like model systems. We have here
352 tackled this issue, using our DVP approach to generate an in-depth proteome atlas of the
353 homeostatic human colon, which serves as an important reference for future studies assessing
354 e.g. disease-associated changes in the human colon. Notably, the DVP setup does not require
355 fresh tissue dissociation and enrichment of living cells, which reduces the impact of lengthy
356 isolation protocols on cellular phenotypes and thereby enabled us to assess the proteomes of
357 mucosal cell types in their native state. We successfully identified and differentiated the isolated
358 mucosal cell populations. Interestingly, aside from cell type-specific protein abundance patterns,
359 we observed location-skewed protein abundance along the mucosal crypt axis. A similar zonation
360 has been reported previously for murine small intestinal epithelial cells at transcriptome level¹⁴,
361 and it is well known that differences in e.g. WNT and BMP signaling along the crypt axis regulate
362 epithelial phenotypes¹. We here address this comprehensively across the different cell types in the
363 mucosa at the protein level and identify the proteins ZZEF1 and TAGLN, which associate with the
364 upper or crypt bottom compartment across the analyzed cell types, respectively. In the future, it
365 will be interesting to study this protein regulation along the crypt axis in further detail and to dissect
366 how e.g. WNT and BMP signaling gradients, as well as luminal cues such as microbiota shape
367 protein abundance and cellular identity. This will shed light on the regulatory pathways maintaining
368 tissue structures which are key for intestinal homeostasis and abrogated for example in the context
369 of colorectal cancer^{56,57}.

370 Our human colon proteome atlas further enabled us to benchmark widely used *in vitro*³⁻⁵ and
371 emerging organoid transplantation models¹⁰ for human IECs. Importantly, while we detect robust
372 proteome correlation between IECs grown *in vitro* and their *in vivo* counterparts, which mirrors
373 previous reports on transcriptome level in murine small intestine, we observe a striking phenotypic
374 switch of organoids upon transplantation into the mucosa, rendering them *in vivo*-like. A major
375 difference between organoids grown *in vitro* and transplanted into the mouse colon is a reduction
376 in the proliferation signature, comparable to *in vivo* IECs, upon reintroduction into the mucosa. In

377 addition to the high proliferation state, organoids grown *in vitro* display lower functional features
378 (e.g. ion transport), as well as a different metabolic signature characterized by lower oxidative
379 phosphorylation. In the murine small intestine, oxidative phosphorylation has been linked to the
380 regulation of stem cell identity and differentiation into Paneth cells⁵⁸. We here find that proteins
381 associated with oxidative phosphorylation are, at least in part, differentially regulated depending
382 on epithelial maturation state. It remains to be shown whether this correlates with actual changes
383 in metabolism between epithelial subpopulations, and whether/how epithelial differentiation and
384 metabolism are linked in colonic IECs⁵⁹. We further make use of our dataset to identify CA1 (ion
385 transport/water homeostasis⁴³) and MUC17 (glycocalyx in the brush border of differentiated
386 IECs/barrier function⁴⁴) as markers for human upper crypt IECs *in vivo*.

387 We show that high proliferation and low functional features observed in IECs grown *in vitro* are
388 driven by the culture conditions (high WNT, low BMP signaling), rather than an intrinsic cellular
389 feature selected for during culture, and that this state, including abundance of CA1 and MUC17,
390 can be partially reverted by adjustments in culturing conditions (-WNR +BMP). Notably, recent
391 advances in organoid-on-a-chip models using hydrogels which recapitulate the mucosal crypt
392 structure and molecular gradients, feature similar IEC shifts to a more *in vivo*-like phenotype at
393 transcriptome level⁶⁰. These findings have important implications for the use of organoids to study
394 IEC functions *in vitro*, especially when focusing on the role of upper crypt IECs, e.g. in host-microbe
395 interactions.

396 The phenotypic reversion of organoids transplanted into the murine colon to a more *in vivo*-like
397 phenotype highlights a remarkable homology between mouse and human stem cell niche factors.
398 A more detailed analysis of the differences between transplanted organoids and IECs *in vivo* will
399 reveal which molecular pathways drive the difference we observed between these two populations.
400 One key aspect aside from the limited compatibility of mouse and human growth factor signaling
401 could be the fact that we used immunocompromised mice for the xenotransplantation to prevent
402 rejection. Future studies comparing human to murine organoids transplanted into the murine colon
403 will be able to dissect the impact of species-specificity and the presence of immune cells on
404 transplanted epithelial cells.

405 Finally, we capitalize on the unprecedented possibility to characterize human LGR5⁺ stem cells
406 in the colon mucosa to identify a human stem cell proteome signature, which reveals EPHB3,
407 MRE11 and MCM2 as antibody-stainable markers for the enrichment of human colonic stem cells
408 *in vivo*. Notably, expression of these markers is more strongly restricted to the crypt bottom *in vivo*
409 compared to previously published markers for the enrichment of human stem cells (EPHB2, PTK7,
410 OLFM4). As EPHB3 is a surface protein, we expect that this marker will be of great value for the
411 community to identify and isolate stem cell-enriched IECs for future studies of human intestinal
412 stem cells. Furthermore, this showcases the strength of DVP to i) efficiently isolate rare cells from
413 tissue in their native state and to ii) use proteome data to directly identify antibody-stainable
414 markers. In addition, it serves as a proof-of-principle for the specific isolation and analysis of

415 genetically modified xenotransplanted human IECs from the murine colon and lays the base for
416 future mechanistic studies, e.g. in the context of tissue damage and repair, and host-microbe
417 interactions.

418 We here advanced the DVP pipeline, demonstrating that DVP is a uniquely well-suited
419 methodology for the faithful in-depth analysis of functional cellular phenotypes in a densely packed
420 tissue like the colon mucosa. Limited sensitivity has so far been a major difficulty for the use of
421 proteomics to dissect dynamic tissue processes, especially in the context of tightly regulated
422 responses such as inflammation (i.e., low abundant, spatially restricted proteins). An additional
423 limitation has been the ability to isolate cells in a near to native state, in the absence of alterations
424 by tissue handling including single cell isolation. The DVP protocol we use here tackles these
425 hurdles, enabling higher throughput and requiring less input material than the original method, and
426 preserving spatial context while reducing the impact of isolation protocols on cellular phenotypes.
427 These technological advancements are promising regarding the expansion of DVP for the
428 acquisition of proteomes of single cells⁶¹. This opens exciting perspectives for the use of DVP to
429 study dynamic tissue processes such as inflammation, even from rare patient material.

430 Taken together, the presented data has important implications for the selection of *in vitro*
431 organoid systems to study specific aspects of epithelial cell biology. The phenotypic reversion of
432 organoids transplanted into the murine colon to a more *in vivo*-like phenotype highlights the
433 impressive homology between mouse and human stem cell niche factors, underlines the suitability
434 of the murine (orthotopic transplantation) model for studies of epithelial-niche interactions with a
435 translational perspective and opens exciting possibilities for the use of organoid transplantation in
436 regenerative medicine.

437

438 **Methods**

439 **Human colon mucosa samples**

440 All individuals included in this study were attending the Department of Gastroenterology, Herlev
441 Hospital, University of Copenhagen, Denmark, for the Danish National Screening Program for
442 Colorectal Cancer or evaluated for various gastrointestinal symptoms but were included only if all
443 subsequent examinations were normal. The exclusion criteria included age below 18 or over 80
444 years; impaired cognitive functions, e.g., dementia; pregnant or lactation women; ongoing
445 treatment with anticoagulation, and patients unable to understand Danish language. The study
446 was approved by the Scientific Ethics Committee of the Capital Region of Denmark (reg. no. H-
447 21038375). All individuals were informed of the study both orally and in writing, in compliance with
448 the Declaration of Helsinki and the guidelines of the Danish National Scientific Ethics Committee.
449 Written informed consent was obtained prior to inclusion.

450 For those individuals included, human colon mucosa samples (cancer-associated bowel
451 resection or biopsies (healthy individuals undergoing cancer screening)) were immediately
452 transferred to 4% PFA (Sigma) upon sampling and fixed at 4 °C for 2-10 days, depending on

453 sample size. Samples were then washed in PBS and transferred to 30% sucrose/PBS and
454 dehydrated for 2-10 days at 4 °C. Next, samples were embedded in OCT, frozen on dry ice and
455 stored at -80C until further analysis.

456

457 **Human colon organoid culture**

458 Human colon organoids were cultured as previously described⁵. Briefly, upon single cell
459 dissociation, 3,000 – 4,000 single cells were seeded in 30 µL Matrigel domes and maintained in
460 advanced DMEM/F-12, supplemented with penicillin-streptomycin, 10 mM HEPES, 2 mM
461 GlutaMAX, 100 ng/mL recombinant mouse Noggin, 1x B27, 500 nM A83-01, 1% NGS-WNT, 1
462 mg/mL recombinant human R-spondin-1, 100 ng/mL recombinant human IGF, 50 ng/mL
463 recombinant human FGF2, 1 mM N-Acetylcysteine and 10 nM recombinant human Gastrin. For
464 WNR withdrawal, organoids were cultured in conventional medium until d7. Organoids were then
465 reseeded in fresh Matrigel domes (no splitting) and maintained until d10 in advanced DMEM/F-12,
466 supplemented with penicillin-streptomycin (Penstrep), 10 mM HEPES, 2 mM GlutaMAX, 1x B27,
467 500 nM A83-01, 100 ng/mL recombinant human IGF, 50 ng/mL recombinant human FGF2, 1 mM
468 N-Acetylcysteine and 10 nM recombinant human Gastrin in the presence or absence of BMP4 (10
469 ng/ml). Organoids were split every 7d for maintenance. Organoids were harvested at d10 for the
470 analyses presented in this study. Human colon organoids from healthy individuals have been used
471 for this study. The LGR5-TdTomato reporter organoid line has been described before¹². To
472 introduce a constitutive GFP reporter to the cells for easier localization of the transplant, eight wells
473 (i.e. eight 30 µL Matrigel domes) of organoids were mechanically disrupted, washed and
474 resuspended ~600 µL media supplemented with Y-27632 (10 uM). Lenti virus was added to the
475 cells to transduce them with a plasmid expressing GFP under the SFFV promotor⁶². The cells were
476 incubated for 4 h at 37 °C, washed three times in DMEM medium and subsequently seeded into
477 four Matrigel domes (30 µL). After three days of culture, transduced cells were selected by addition
478 of 2 ug/ml Puromycin to the media. Cells were passaged twice, tested according to FELASA
479 standards (IDEXX), and subsequently used for transplantation.

480 For cryosamples, 500 µL ice cold cell recovery solution was added to each well. Matrigel domes
481 were carefully scraped off with a cut open P1000 pipet tip and transferred to 5 ml cell recovery
482 solution (R&D systems) on ice. After 30 min, the supernatant was removed, organoids were
483 resuspended in 4% PFA and fixed for 1h at ambient temperature. Subsequently, organoids were
484 washed three times in 5 ml PBS (if necessary, organoids were spun down for 2 min at 100g),
485 embedded in OCT (Tissue Tek) in cryomolds, and frozen on dry ice. Samples were stored at -80 °C
486 until further analysis.

487 For bulk proteome analysis, organoids were harvested as previously described⁶³. Briefly, 1 ml
488 ice cold 0.1% BSA/PBS was added to each well and matrigel domes were broken up by pipetting
489 10 times with a P1000 pipet. Organoids from four wells were pooled per sample in a tube containing
490 3 ml 0.1% BSA/PBS. Cells were pelleted by centrifugation (5 min, 300 x g, 4 °C), supernatant was

491 removed and cells were resuspended in 1 ml 0.1% BSA/PBS and pelleted again. Upon removal of
492 the supernatant, cells were resuspended in 200 µL 0.1% BSA/PBS and transferred to a 1.5 ml
493 Eppendorf tube (pre-coated with 0.1% BSA/PBS) and kept on ice until further processing.
494

495 **Orthotopic xenotransplantation**

496 NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug}/JicTac (NOG) mice were used for transplantation assays. All
497 animal procedures were approved by the Danish Animal Inspectorate (license number 2018-15-
498 0201-01569 to Kim B. Jensen).

499 In preparation of the transplantation, organoids were grown as described above until d5-6 in 6-
500 well plates containing nine Matrigel domes per well. 3 ml ice cold cell recovery solution was added
501 to each well. Matrigel domes were carefully scraped off with a cut open P1000 pipet tip and
502 transferred to 5 ml cell recovery solution (R&D systems) on ice for 20 min. Cells were subsequently
503 pelleted for 3 min at 300 x g, washed once in PBS and resuspended in 200 µL of 5% Matrigel/PBS
504 per mouse. Right before transplantation, organoids were dissociated by pipetting 20x with a pre-
505 wet P1000 pipette.

506 Transplantation was performed as described previously¹¹, with slight modifications. Mice were
507 anesthetized with 2% isoflurane before the procedure. The colon content was flushed with PBS
508 and an electric interdental brush, soaked in prewarmed 0.5 M EDTA, was used to brush crypts off
509 on one side of the colon. The organoids suspension was subsequently infused into the conditioned
510 colon. Glue (Histo-acryl, B. Braun) was added to the anal verge and left for 3h to avoid the ejection
511 of the organoid suspension and thereby enhance the engraftment of the infused material. Mice
512 were monitored daily. Transplanted samples were isolated six weeks after transplantation. For
513 cryosectioning, the colon was isolated, cut open and placed under a fluorescent microscope (Evos)
514 to locate GFP⁺ transplanted cells. The colon area containing the transplant was subsequently cut
515 out, fixed in 4% PFA at 4 °C over night, dehydrated in 30% sucrose/PBS over night at 4 °C and
516 then embedded in OCT and frozen on dry ice. Samples were kept at -80 °C until further analysis.
517

518 **Cryosectioning, immunofluorescent staining and imaging for DVP**

519 2-mm-thick polyethylene naphthalate membrane slides (Zeiss) were pretreated by ultraviolet
520 ionization for 3 h. Without delay, slides were consecutively washed for 5 min each in 350 ml
521 acetone and 7 ml VECTABOND reagent to 350 ml with acetone, and then washed in ultrapure
522 water for 30 s before drying in a gentle nitrogen air flow. The slides were treated with a dilution of
523 7 mL Vectabond in 350 mL acetone for 5 minutes without prior washing in acetone or subsequent
524 washing in water. Afterwards, the slides were dried in an incubator at 30 °C for 3 hours.

525 Frozen samples in OCT were cut with a Leica cryostat in 5 um sections. Samples were
526 subsequently dried for 1h at ambient temperature, rehydrated with 500 µL PBS for 1 min and
527 permeabilized with 300 ul PBS/0.5% TritonX-100. Tissue sections were blocked in 200 µL
528 PBS/donkey serum for 30 min at room temperature and subsequently incubated with the primary

529 antibody mix in blocking buffer overnight at 4C. The next day, samples were washed three times
530 with 500 µL PBS and incubated for 40 min at ambient temperature with the secondary antibody
531 mix in PBS. Upon washing three times with PBS, samples were mounted using anti-fade
532 fluorescence mounting medium (abcam). Samples were subsequently imaged as described below
533 and, if necessary subjected to a second round of staining. For this, samples were bleached using
534 bleaching buffer (24 mM NaOH and 4.5% H₂O₂) for 10 min at room temperature, washed with
535 PBS and stained as above.

536 Antibodies and staining reagents used in this study: CD45-BV421 (30-F11, Biolegend, 1:100),
537 Lrig1 (R&D Systems AF3688, 1:50), PDGFR (EPR22059-270, abcam, 1:100), UEA-Atto550 (Atto-
538 Tec, 1:500), EPCAM-APC (EBA1, BD Biosciences, 1:50), EPCAM-APC (G8.8, Fisher
539 Scientific, 1:50), ECAD (ECCD2, Thermo Fisher, 1:200), CD45 (HI30, Stem cell, 1:200), DAPI
540 (Sigma), MUC17 (Merck HPA031634, 1:200), CA1 (EPR5193, abcam, 1:200), Pan-Laminin-AF647
541 (Novus Biologicals NB300-144AF647, 1:100).

542 The samples were imaged on a Zeiss AxioScan 7 microscope slide scanner at a magnification
543 of 20×, with three z-layers with intervals of 2.5 mm. Human colon tissues were imaged in two
544 consecutive rounds. For the first round, the acquisition settings were 4 ms illumination time and 1.49%
545 385 nm laser for DAPI, 20 ms illumination time and 100% 475 nm laser for AF488, and 300 ms
546 illumination time and 100% 735 nm laser for AF750. For the second round, the acquisition settings
547 were 4 ms illumination time and 1.49% 385 nm laser for DAPI, 15 ms illumination time and 100%
548 475 nm laser for AF488, 60 ms illumination time and 100% 567 nm laser for AF568, and 20 ms
549 illumination time and 100% 630 nm laser for AF647. For *in vitro* organoids, the acquisition settings
550 were 2 ms illumination time and 1.1% 385 nm laser for DAPI, 2.2 ms illumination time and 100%
551 475 nm laser for FITC, 30 ms illumination time and 100% 567 nm laser for Rhoda, and 8 ms
552 illumination time and 100% 630nm laser for AF647. Transplanted organoids were imaged in two
553 staining rounds. The first round was imaged with an illumination time of 1.2ms and 1.5% 385 nm
554 laser for DAPI, 3 ms illumination time and 100% 475 nm laser for Af488, 80 ms illumination time
555 and 100% 567 nm laser for tdTomato, 20 ms illumination time and 100% 630 nm laser for Af647,
556 and 100 ms illumination time and 100% 735 nm laser for Af750.

557

558 **Image Analysis**

559 Corresponding images of the two imaging rounds were cropped and subsequently
560 concatenated in imagej. Afterwards, the images were registered using the RigidBody
561 transformation in HyperStackReg on the GFP and tdTomato channel in the transplanted organoids
562 and DAPI in the *in vivo* human colon, and all channels were merged.

563 Images were split into tiles using the Biological Image Analysis Software (BIAS , Single-Cell
564 Technologies Ltd.) and each tile was segmented in Napari using the cellpose cytosolic algorithm
565 in the serialcellpose plugin. Images were not treated as RGB, batch size was set to 3, flow
566 threshold was set to 3, cell probability threshold was set to -4, diameter was set to 30, the magenta

567 channel was set as channel to segment, and the yellow channel was used as a helper channel.
568 Image analysis was continued in BIAS by filtering shapes for a minimum size of 50 μm^2 and a
569 maximum size of 2000 μm^2 . Features of segmented cells were extracted and classified using a
570 multi-layer perceptron classifier with default settings. For human colon tissue, the bottom part of
571 crypts was manually annotated using the region feature to distinguish stem cells and differentiated
572 epithelium. Contours of cells were sorted using the “Greedy” setting and coordinates of the
573 contours were exported.

574

575 **Laser Microdissection**

576 Contours were imported at 63 \times magnification, and laser microdissection performed with the
577 LMD7 (Leica) in a semi-automated manner at the following settings: power 46, aperture 1, speed
578 40, middle pulse count 4, final pulse 8, head current 46-50%, and pulse frequency 2,600. Contours
579 were sorted into a low-binding 384-well plate (Eppendorf 0030129547). 500 contours were
580 collected per sample except for immune cells surrounding upper crypt of which 700 contours were
581 collected. Due to limited sample amount in the transplanted organoids, 200 contours were
582 collected for differentiated cells and about 100 contours were collected for stem cells. An overview
583 of collected biological replicates and technical replicates per cell population can be found in the
584 supplementary data (Table S1). Contours were rinsed to the bottom of the well by filling the wells
585 up with 40 mL acetonitrile, vortexing for 10 seconds, and centrifuging at 2000 $\times g$ at ambient
586 temperature for 5 min. A SpeedVac was used to evaporate the acetonitrile at 60 °C for 20 min or
587 until achieving complete dryness and the contours were stored at 4 °C.

588

589 **DVP proteome sample preparation and acquisition**

590 Lysis was performed in 4 mL of 0.01 % n-dodecyl-beta-maltoside in 60 mM triethyl ammonium
591 bicarbonate (TEAB, pH 8.5, Sigma) at 95 °C in a PCR cycler with a lid temperature of 110 °C for 1
592 h. 1 mL of 60% acetonitrile in 60 mM TEAB was added and lysis continued at 75 °C for 1 h. Proteins
593 were first digested with 4 ng LysC at 37 °C for 3 h and subsequently digested overnight using 6 ng
594 trypsin at 37 °C. The digestion was terminated by adding 1.5 mL 5 % TFA. Samples were dried in
595 a SpeedVac at 60 °C for 40 min and stored at -80 °C.

596 C-18 tips (Evotip Pure, EvoSep) were washed with 100 μL of buffer B (0.1% formic acid in
597 acetonitrile), activated for 1 min in 1-propanol, and washed once with 20 μL buffer A (0.1% formic
598 acid). Samples were resuspended in 20 mL buffer A on a thermoshaker at room temperature at
599 700 $\times g$ for 15 min. Peptides were loaded on the C-18 tips, washed with 20 mL buffer A, and then
600 topped up with 100 mL buffer A. All centrifugation steps were performed at 700 $\times g$ for 1 min, except
601 peptide loading at 800 $\times g$ for 1 min.

602 Samples were measured with the Evosep One LC system (EvoSep) coupled to an Orbitrap
603 Astral mass spectrometer (Thermo Fisher). Peptides were separated on an Aurora Elite column
604 (15 cm x 75 mm ID with 1.7 mm media, IonOpticks) at 40 °C running the Whisper40 gradient. The

605 mobile phases were 0.1% formic acid in liquid chromatography (LC)–MS-grade water (buffer A)
606 and 0.1% formic acid in acetonitrile (buffer B). For samples consisting of 500 contours, the Orbitrap
607 Astral MS was operated at a full MS resolution of 240,000 with a full scan range of 380 – 980 m/z.
608 The AGC target was set to 500% for full scans and fragment ion scans. Fragment ion scans were
609 recorded with a maximum injection time of 5 ms and with 300 windows of 2 Th scanning from 150
610 – 2000 m/z. Fragmentation of precursor ions took place using HCD with 25% NCE. Samples
611 consisting of 200 contours (stem cells from transplanted organoids) were acquired using a full
612 maximum injection time of 100 ms for MS1. Fragment ion scans were recorded with a maximum
613 injection time of 14 ms (MS2), an AGC target of 800 %, and with 75 windows of 8 Th scanning
614 from 150 – 2000 m/z.

615

616 **DVP raw MS data analysis**

617 Raw files were converted to mzML using MSconvert and analyzed in DIA-NN 1.8.1 using an in-
618 silico DIA-NN predicted spectral library (101370 protein isoforms, 177027 protein groups and
619 7821224 precursors in 3872218 elution groups)⁶⁴. A human proteome reference database,
620 including isoform information and the tdTomato fluorophore sequence, was used to generate the
621 library and search the raw files (Uniprot March 2023). Following configuration was set for the
622 search: N-terminal methionine excision was enabled, digest was performed at K* and R*,
623 maximum number of missed cleavages was set to 2, maximum number of variable modifications
624 was set to 2, oxidation of methionine was considered as variable, acetylation of the N-terminus
625 was considered as variable, Protein inference = “Genes”, Neural network classifier = “Single-pass
626 mode”, Quantification strategy = “Robust LC(high precision)”, Cross-run normalization = “RT-
627 dependent”, Library Generation = “Smart profiling”, and Speed and RAM usage = “Optimal results”.
628 Mass accuracy and MS1 accuracy were set to 15. “Use isotopologues”, “No shared spectra”,
629 “Heuristic protein inference” and “MBR” were activated.

630

631 **DVP data analysis**

632 Data analysis was mostly performed in Perseus and AlphaPeptStats^{65,66}. Python and R were
633 used to conduct further analyses and visualize the data. The first technical replicate of the second
634 biological replicate of fibroblasts at the bottom of crypts (fib_top_02_01) was removed due to the
635 quantification of less than 2000 proteins. Raw data was imported into Perseus, and proteins filtered
636 for 80 % data completeness within samples of the same cell type and same location in the human
637 tissue. Missing values were replaced from a normal distribution with a width of 0.3 and a down shift
638 of 1.3. Data was normalized by aligning the median intensity of all samples. Median intensities of
639 each sample were determined, and the median of these median intensities was divided by the
640 median of each sample. The resulting factor was multiplied with each intensity of the sample.
641 Differential abundance analyses for volcano plots and enrichment analyses were performed in
642 Perseus. Kruskal-Wallis tests were performed in Perseus with Benjamini-Hochberg FDR correction

643 and a threshold of 0.01. GSEAs were performed using the GSEAp (v 1.0.6) package against the
644 GO_Biological_Process_2023 dataset^{67,68}.

645

646

647 **Bulk Proteome sample preparation and acquisition**

648 200 mL 60 mM TEAB lysis buffer was added to the washed and pelleted organoids. Samples
649 were lysed at 95 °C shaking at 800 rpm for 30 min. Afterwards the lysate was sonicated at 4 °C in
650 30 s intervals for 10 min. 18 mL ACN was added to bring the lysis buffer to a final concentration of
651 12.5 % ACN and lysis continued at 95 °C shaking at 800 rpm for another 30 min. Debris was
652 pelleted at 4 °C at 20,000 x g for 10 min and supernatants transferred to fresh tubes. Protein
653 concentration of supernatants was determined using nanodrop and 200 mg were used for further
654 processing. Lys-C and trypsin were added at a protein to enzyme ratio of 50:1. Digestion took
655 place at 37 °C shaking at 800 rpm overnight. Peptides were lyophilized using a SpeedVac at 60
656 °C for 1 hour. Peptides were resuspended in 200 mL Evosep buffer A (0.1 % formic acid) and 60
657 mL corresponding to 60 mg were loaded in triplicates on 3 layers of SDB-RPS membranes. About
658 10 ng were loaded on Evtips Pure.

659 Samples were measured with the Evosep One LC system (EvoSep) coupled to an Orbitrap
660 Astral mass spectrometer (Thermo Fisher). Peptides were separated on an Aurora Elite column
661 (15 cm x 75 mm ID with 1.7 mm media, IonOpticks) at 40 °C running the Whisper40 gradient. The
662 mobile phases were 0.1% formic acid in liquid chromatography (LC)–MS-grade water (buffer A)
663 and 0.1% formic acid in acetonitrile (buffer B). The Orbitrap Astral MS was operated at a full MS
664 resolution of 240,000 with a full scan range of 380 – 980 m/z and a maximum injection time of 100
665 ms. The AGC target was set to 500% for full scans and fragment ion scans. Fragment ion scans
666 were recorded with a maximum injection time of 5 ms and with 300 windows of 2 Th scanning from
667 150 – 2000 m/z. Fragmentation of precursor ions took place using HCD with 25% NCE.

668

669 **Bulk proteome raw MS data analysis**

670 Raw files were converted to mzML using MSconvert and analyzed together with the DVP
671 samples in DIA-NN 1.8.1 using an in-silico DIA-NN predicted spectral library (101370 protein
672 isoforms, 177027 protein groups and 7821224 precursors in 3872218 elution groups)^{64,69}. A human
673 proteome reference database, including isoform information and the tdTomato fluorophore
674 sequence, was used to generate the library and search the raw files (Uniprot March 2023).
675 Following configuration was set for the search: N-terminal methionine excision was enabled, digest
676 was performed at K* and R*, maximum number of missed cleavages was set to 2, maximum
677 number of variable modifications was set to 2, oxidation of methionine was considered as variable,
678 acetylation of the N-terminus was considered as variable, Protein inference = “Genes”, Neural
679 network classifier = “Single-pass mode”, Quantification strategy = “Robust LC(high precision)”,
680 Cross-run normalization = “RT-dependent”, Library Generation = “Smart profiling”, and Speed and

681 RAM usage = “Optimal results”. Mass accuracy and MS1 accuracy were set to 15. “Use
682 isotopologues”, “No shared spectra”, “Heuristic protein inference” and “MBR” were activated.

683

684 **Bulk proteome data analysis**

685 Data analysis was mostly performed in Perseus and AlphaPeptStats. Python and R were used
686 to conduct further analyses and visualize the data. Raw data was imported into Perseus, and
687 proteins filtered for 80 % data completeness within samples of the same cell type and same
688 location in the human tissue. Missing values were replaced from a normal distribution with a width
689 of 0.3 and a down shift of 1.3. Differential abundance analyses for volcano plots and enrichment
690 analyses were performed in Perseus and visualized in python and R. GSEAs were performed using
691 the GSEAp (v 1.0.6) package against the GO_Biological_Process_2023 dataset.

692

693 **Author contributions**

694 Conceptualization: FP, AH, AM, KJB, MM. Experimentation: FP, AH, SK, SS, AB, DLC, JS.
695 Reagents and material: TLB, TS, CS, OHN. Writing – original draft: FP, AH, KJB, MM. Writing –
696 review and editing: all authors.

697

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710

711 **Conflicts of interest**

712 CS lectures for MSD and Janssen-Cilag and received a research grant from Takeda. MM is an
713 indirect shareholder in Evosep.

714

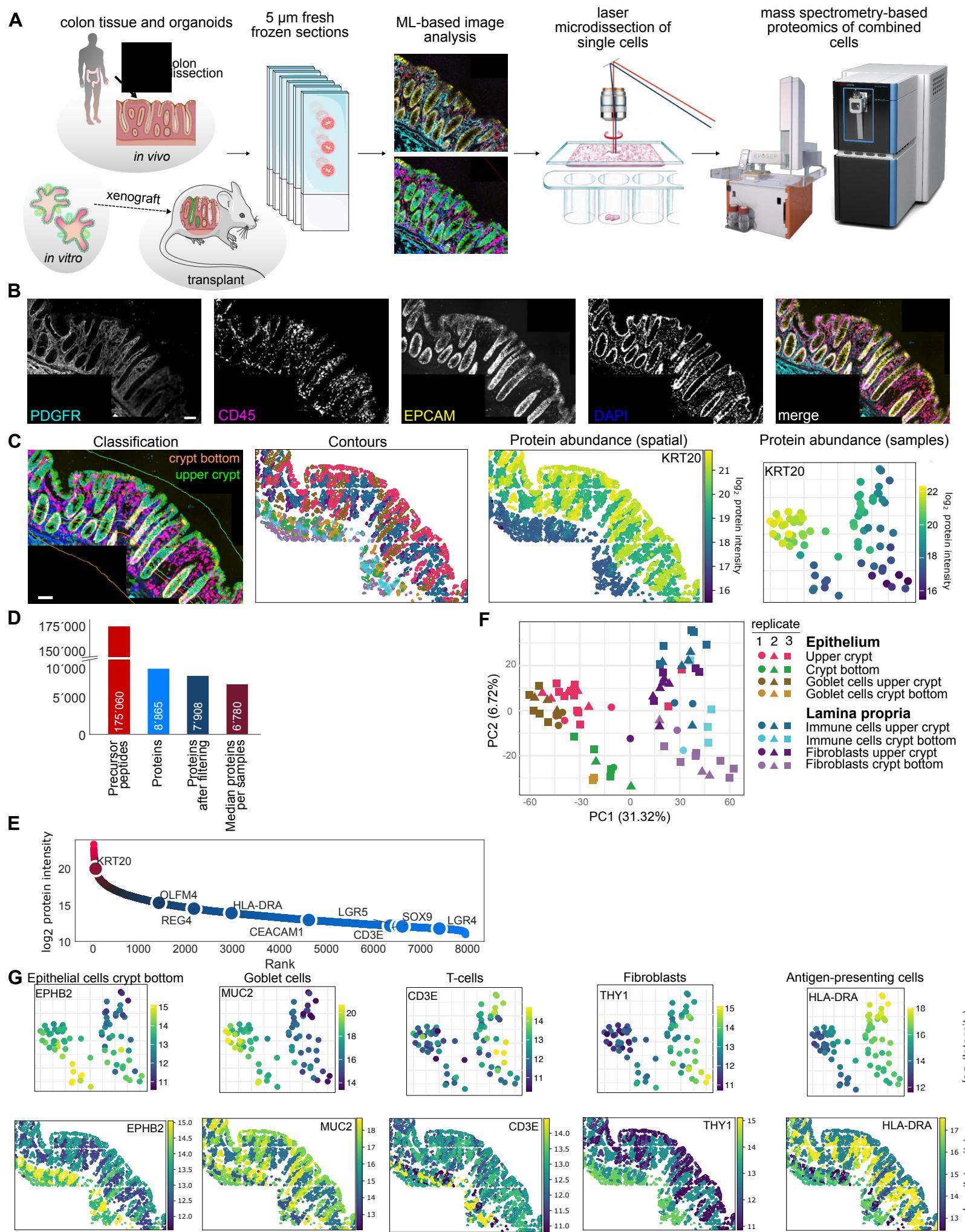


Figure 1

Figure 1: DVP analysis faithfully assesses cellular heterogeneity in the human colon

A Study design for the validation of organoids *in vitro* and organoid transplantation using Deep Visual Proteomics. **B** Immunofluorescence image of the human colon mucosa stained for fibroblasts (PDGFR), immune cells (CD45) and epithelial cells (EPCAM). **C** Crypt bottom and upper crypts were defined by a manually drawn line. Single cells were segmented and classified, contours exported, microdissected, and analyzed. This analysis reveals protein abundance across the colon mucosa and cell populations, as exemplified here for KRT20, a marker of differentiated epithelial cells. **D** Protein and precursor peptide identifications across all samples. **E** Median dynamic range of identified proteins across all samples after imputation and normalization. **F** PCA plot of samples isolated from the colon mucosa (three donors) as indicated by classification in C. **G** Protein abundance and spatial distribution of previously described cell type markers for different subpopulations in the human colon.

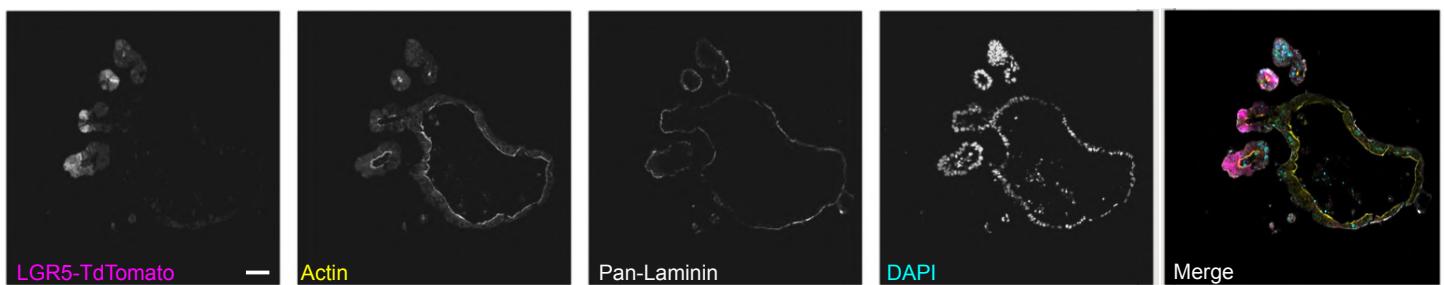
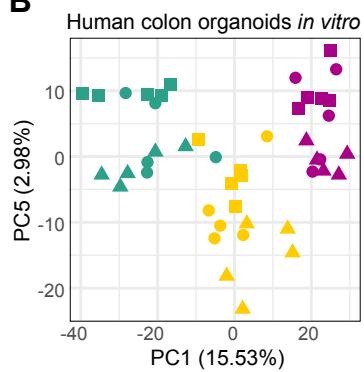
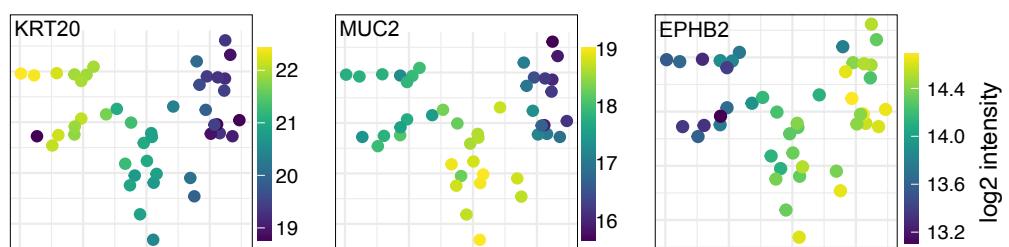
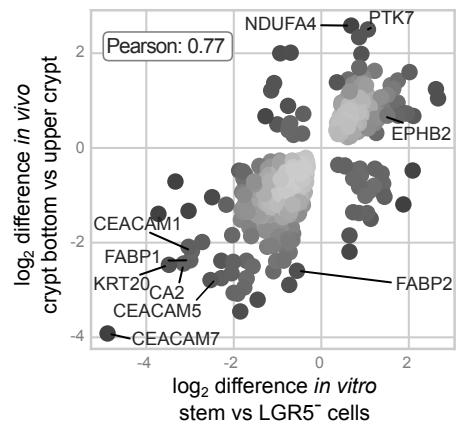
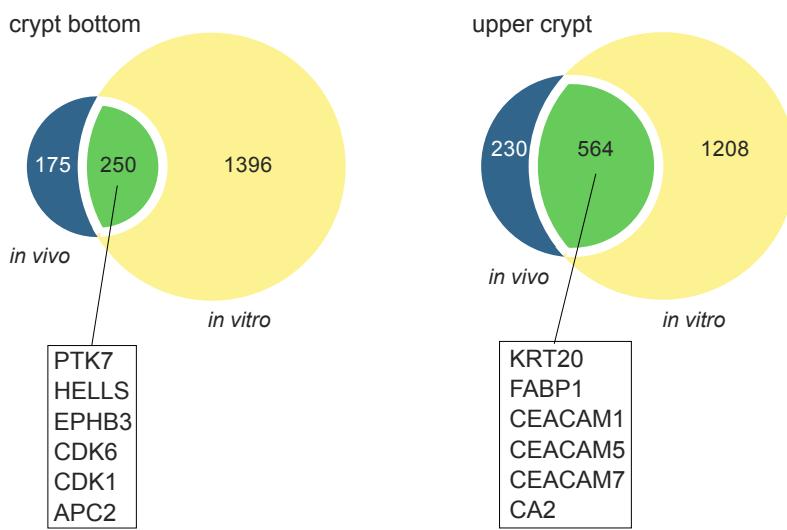
A**B****C****D****E**

Figure 2

Figure 2: DVP analysis reveals a robust correlation between human IECs *in vivo* and grown as organoids

A Immunofluorescence image of a human colon organoid genetically engineered to express TdTomato under an LGR5 reporter for the identification of LGR5⁺ epithelial stem cells. **B** PCA plot of samples isolated from human colon organoids (three biological replicates (one organoid line, three separate passages), five technical replicates). **C** Abundance of previously described markers for different epithelial subpopulations (Krt20 – differentiated epithelial cells, MUC2 – goblet cells, EPHB” – stem cells). **D** Correlation plot of protein intensities of significantly changed proteins in epithelial cells located in the crypt bottom vs upper crypt *in vitro* and *in vivo*. **E** Venn Diagram of significantly changed proteins in epithelial cells in crypt bottom vs upper crypt *in vitro* and *in vivo*. Lines indicate selected overlapping proteins between *in vitro* and *in vivo* crypts.

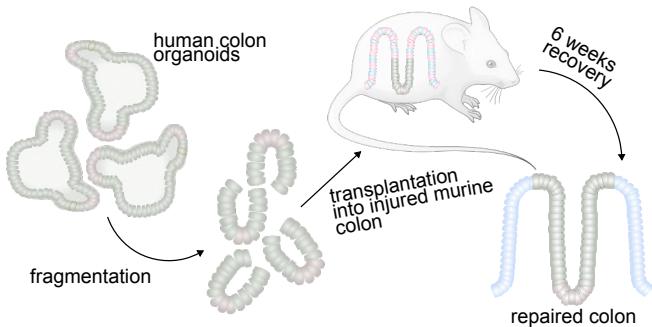
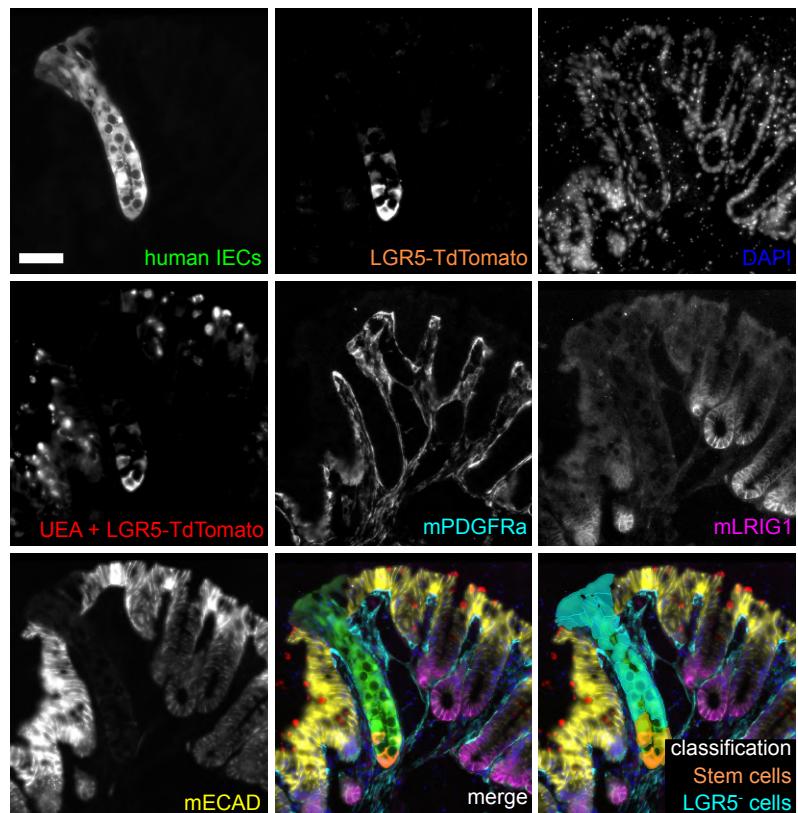
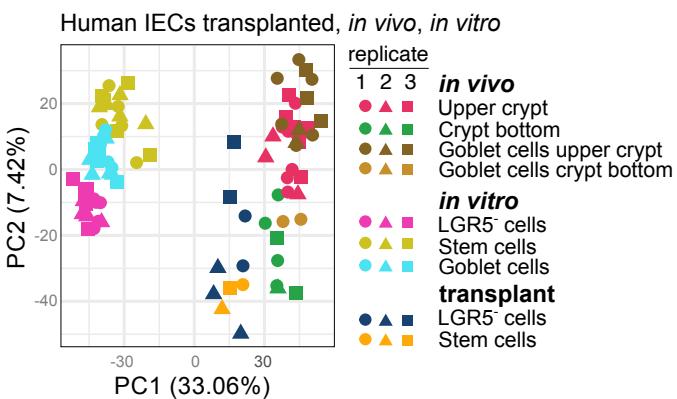
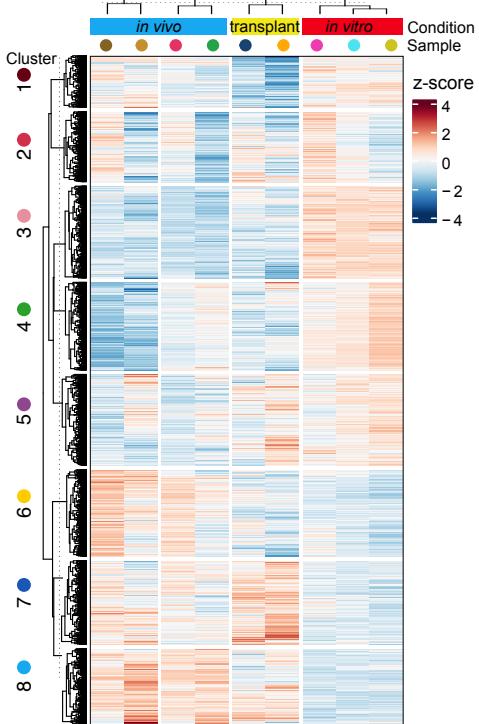
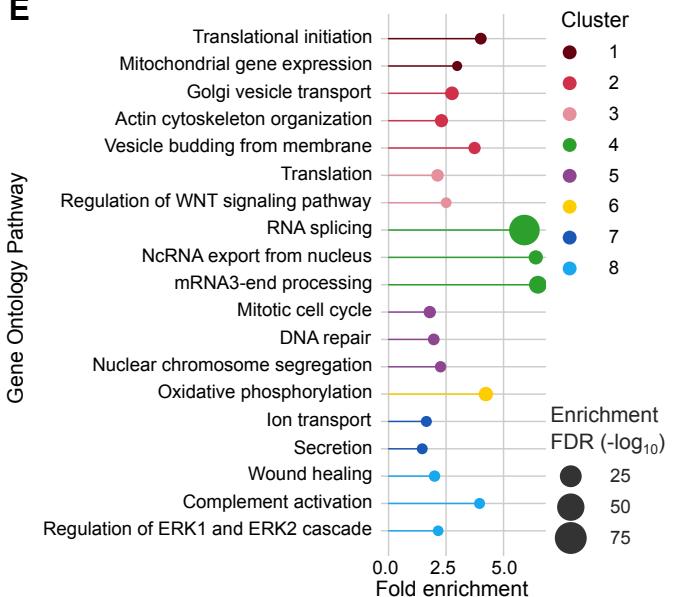
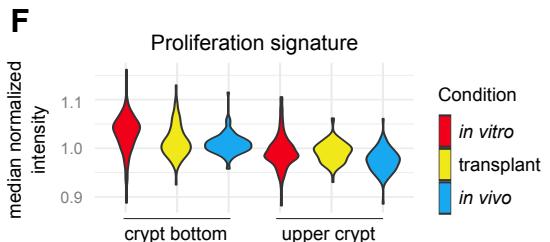
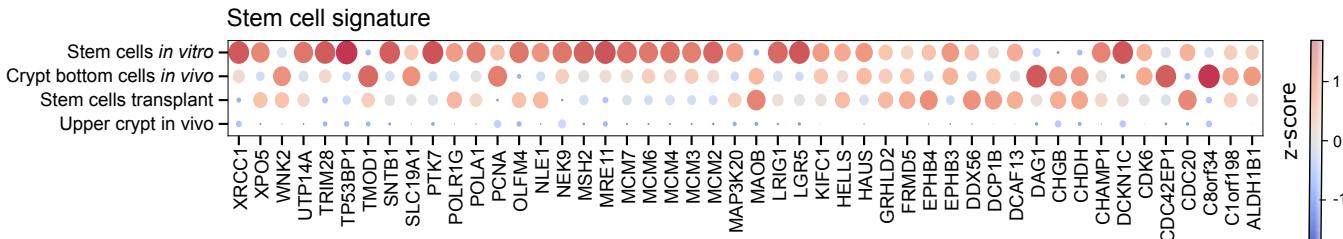
A**B****C****D****E****F****G****Figure 3**

Figure 3: Human colon organoids transplanted into the murine colon recapitulate human colonocytes *in vivo*

A Workflow for orthotopic transplantation of organoids into the murine colon. **B** Immunofluorescence image human colon organoids (Fig. 2) transplanted into the murine colon (transplant). (GFP: human IECs. LGR5: stem cells (human). mECAD: epithelial cells (mouse). mPDGFR: fibroblasts (mouse). mLrig1: crypt bottom compartment (mouse). UEA: mucus (goblet cells). **C** PCA plot of human colonocytes transplanted into the murine colon (one organoid line, three mice, one to three technical replicates), *in vitro* (organoids) and *in vivo* (human colon). **D** Heatmap of significantly changed proteins between organoids *in vitro*, transplanted organoids, and epithelial cells *in vivo*. **E** Gene ontology pathway enrichments of clustered proteins based on the heatmap in 3D. **F** Normalized protein intensities *in vitro*, in transplant, and *in vivo* of proteins that are associated with a proliferation signature in epithelial cells in the crypt bottom and the upper crypt. **G** Human stem cell proteome signature.

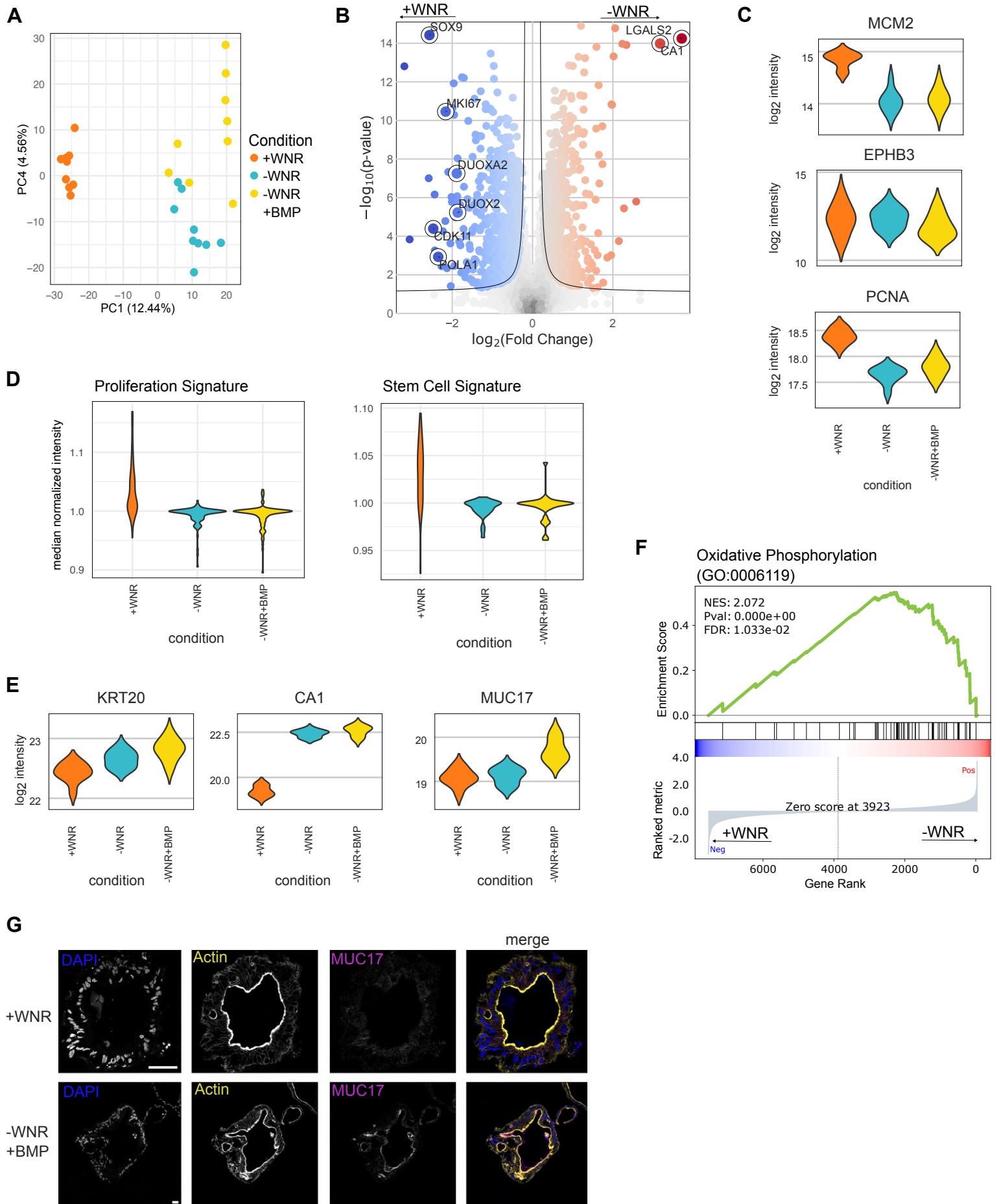


Figure 4

Figure 4: WNR withdrawal in colon organoids cultured *in vitro* induces upregulation of *in vivo* IEC markers.

A PCA of organoids cultured with WNR (WNT3a (W), Noggin (N), R-spondin-3 (R))(+WNR), without WNR (-WNR), and with BMP (Bone Morphogenetic Protein) but without WNR (-WNR +BMP). **B** Volcano plot of organoids cultured with WNR and without WNR. **C** Decrease of stem cell markers of colonic epithelial cells by withdrawal of WNR and addition of BMP. **D** Median normalized intensity of a proliferation signature⁴¹ and stem cell signature in +WNR, -WNR, and -WNR +BMP. **E** Increase of differentiation markers of colonic epithelial cells by withdrawal of WNR and addition of BMP. **F** Fluorescence microscopy showing the increase of MUC17 in colon organoids upon withdrawal of WNR and addition of BMP. **G** Gene Set Enrichment Analysis showing an increase of the oxidative phosphorylation Gene Ontology pathway in -WNR vs +WNR.

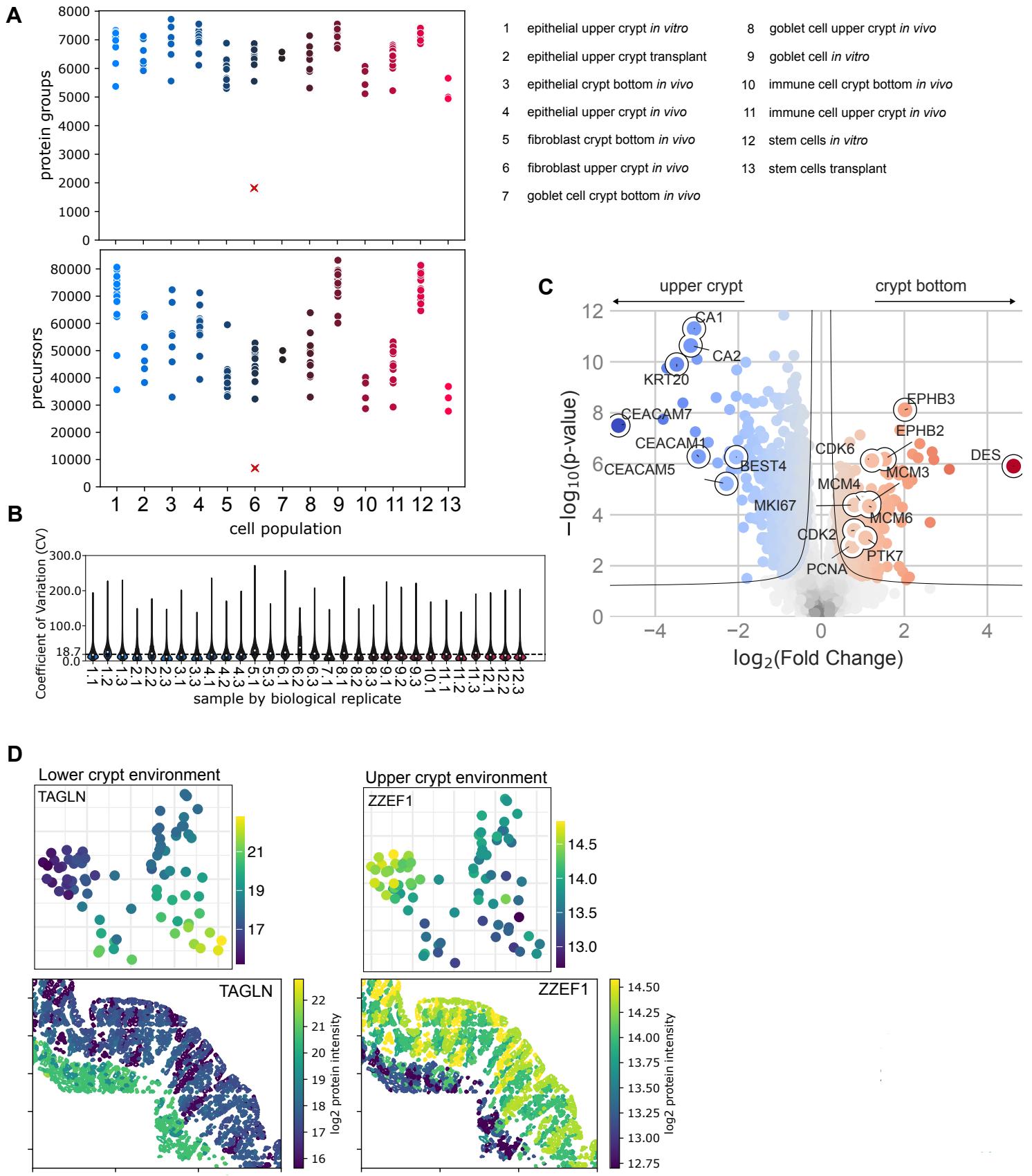


Figure S1

Figure S1

A Number of identified proteins and precursors per sample. **B** Coefficient of variation of technical replicates. **C** Volcano plot comparing epithelial cells from the crypt bottom and upper crypt *in vivo*. **D** Protein abundance and spatial distribution of TAGLN and ZZEF1, which are differentially abundant in the crypt bottom versus upper crypt region in the colon mucosa across different cell types.

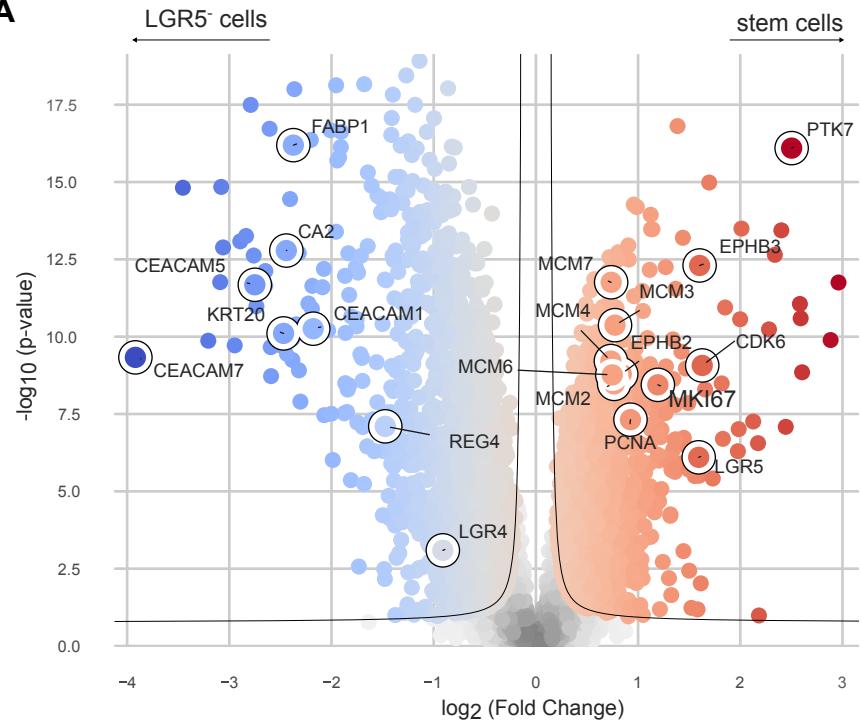
A

Figure S2

Figure S2

A Volcano plot of stem cells (LGR5-TdTomato^+) and LGR5-TdTomato^- cells in organoids *in vitro*.

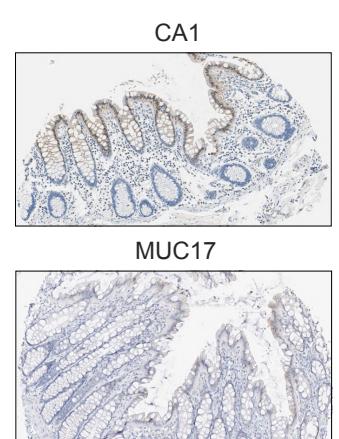
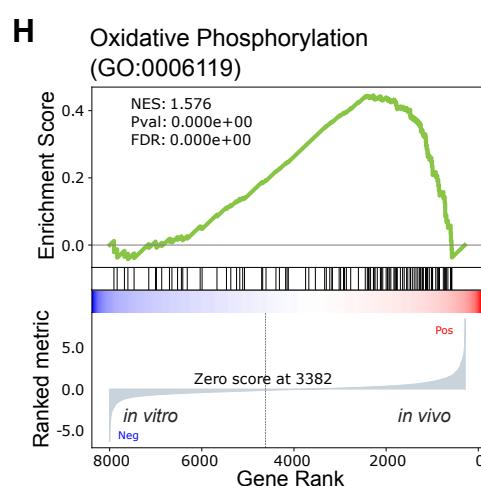
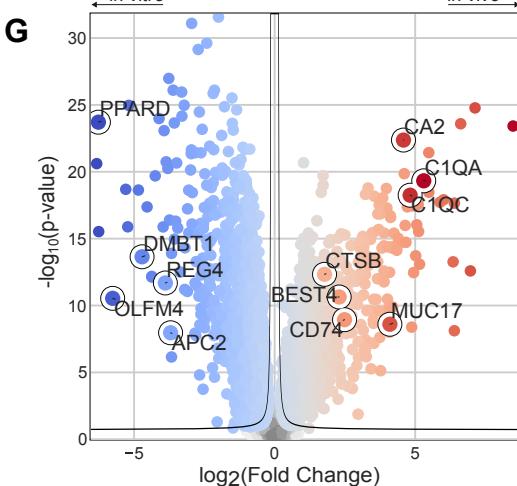
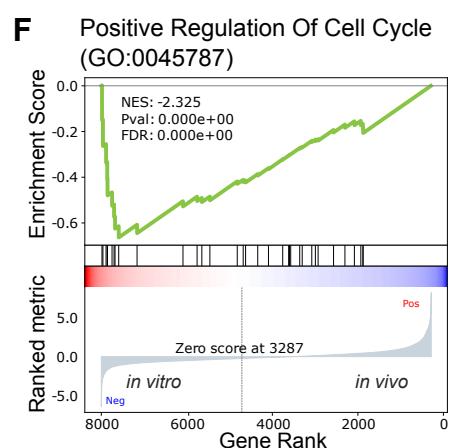
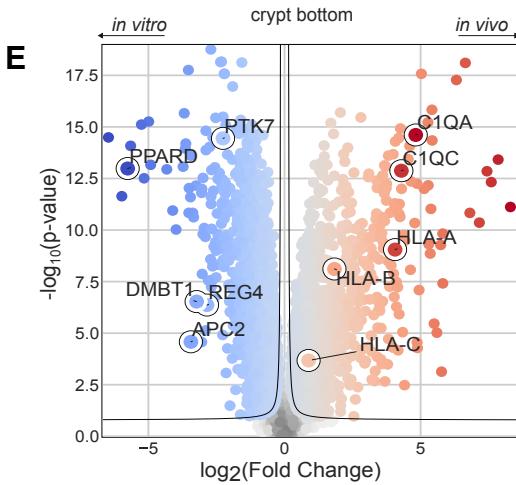
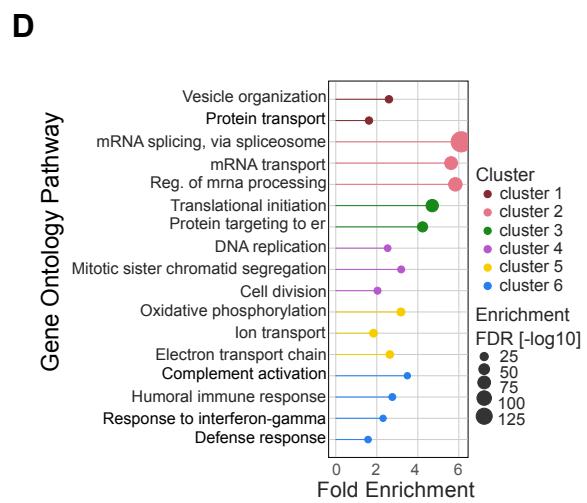
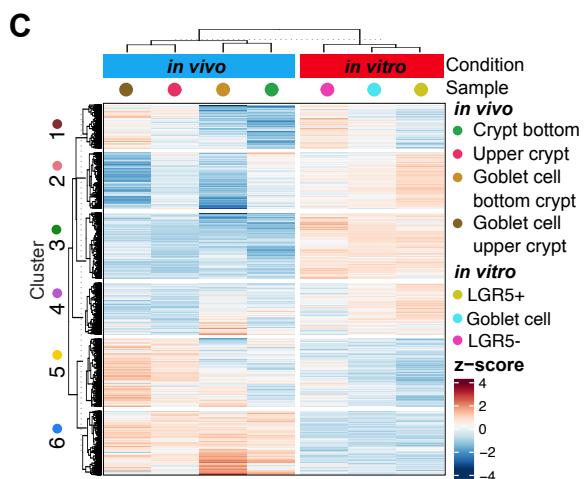
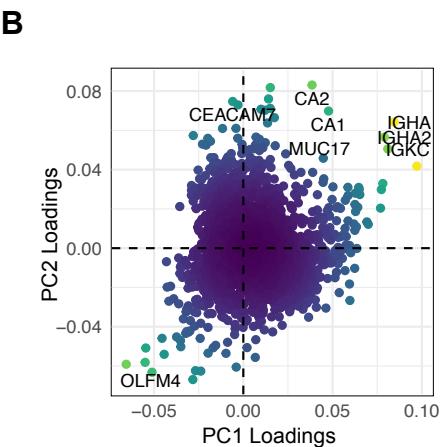
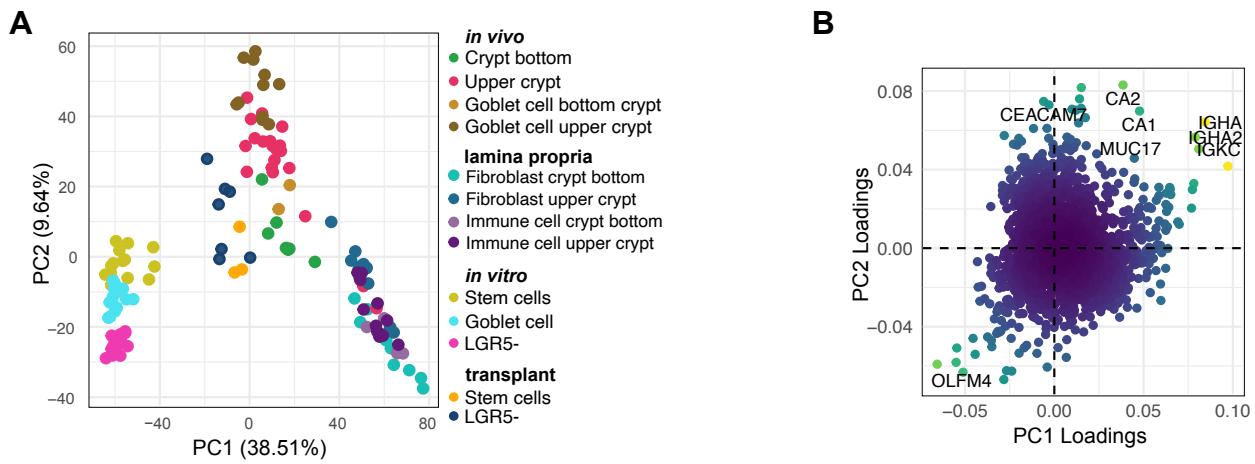


Figure S3

Figure S3

A PCA of the top 3000 most varying proteins across samples in *in vitro*, transplant, and *in vivo*. **B** Loadings describing proteins driving the PCA in S3A. CA1, CA2, MUC17 and CEACAM7 are strongly associated with a crypt top *in vivo* colonocyte phenotype. **C** Heatmap of significantly changed proteins between epithelial cells *in vitro* and *in vivo*. **D** Pathway enrichments of proteins in clusters of Fig S3C. **E** Volcano plot of stem cells *in vitro* vs crypt bottom epithelial cells *in vivo*. **F** Gene Set Enrichment Analysis (GSEA) of the Gene Ontology term “positive regulation of cell cycle” on protein differences of S3E. **G** Volcano plot of epithelial cells in the upper crypt *in vitro* vs *in vivo*. **H** GSEA of the Gene Ontology term “oxidative phosphorylation” on protein differences of S3G. **I** Staining for CA1 and MUC17 in the human colon mucosa from the Human Protein Atlas⁴³.

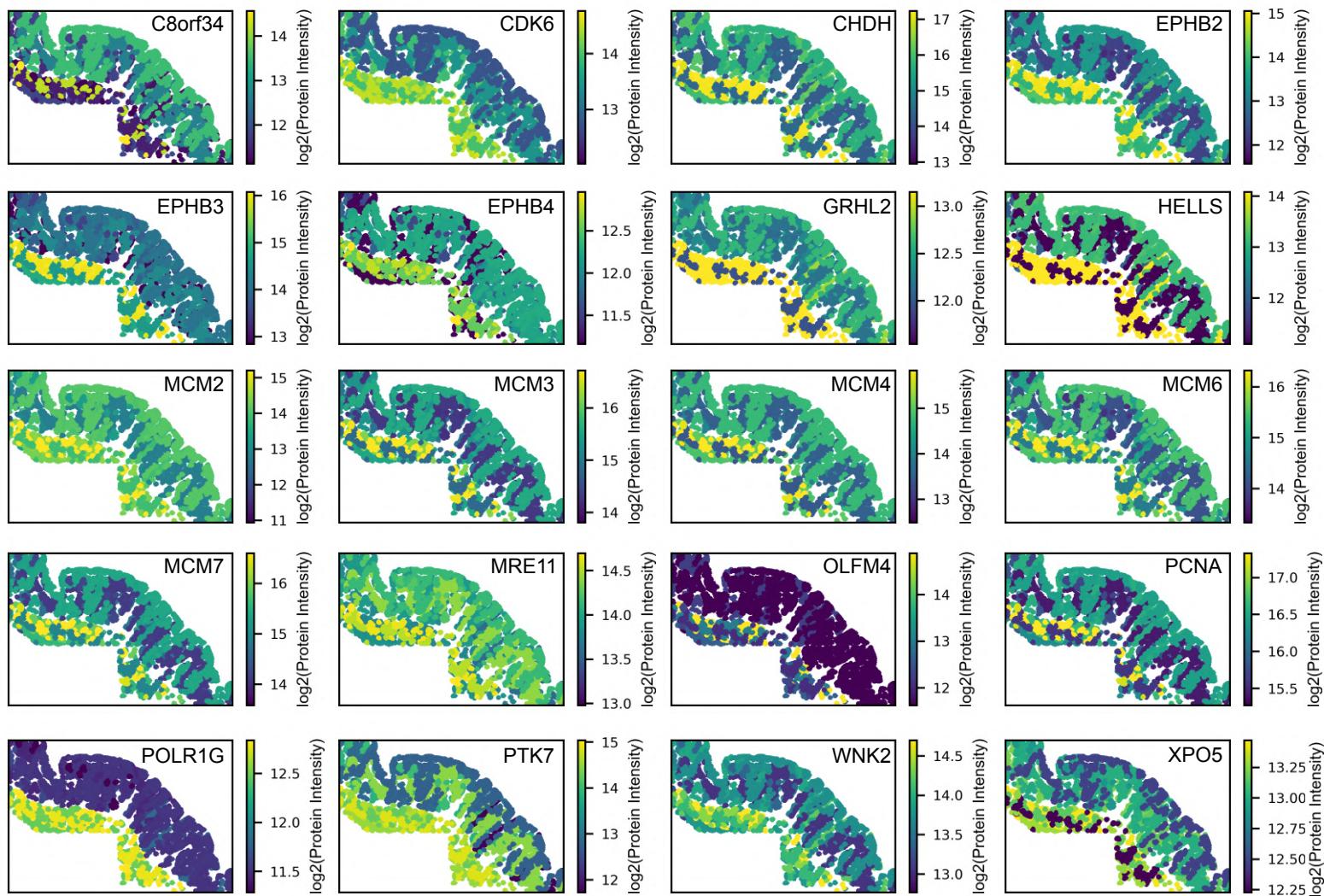
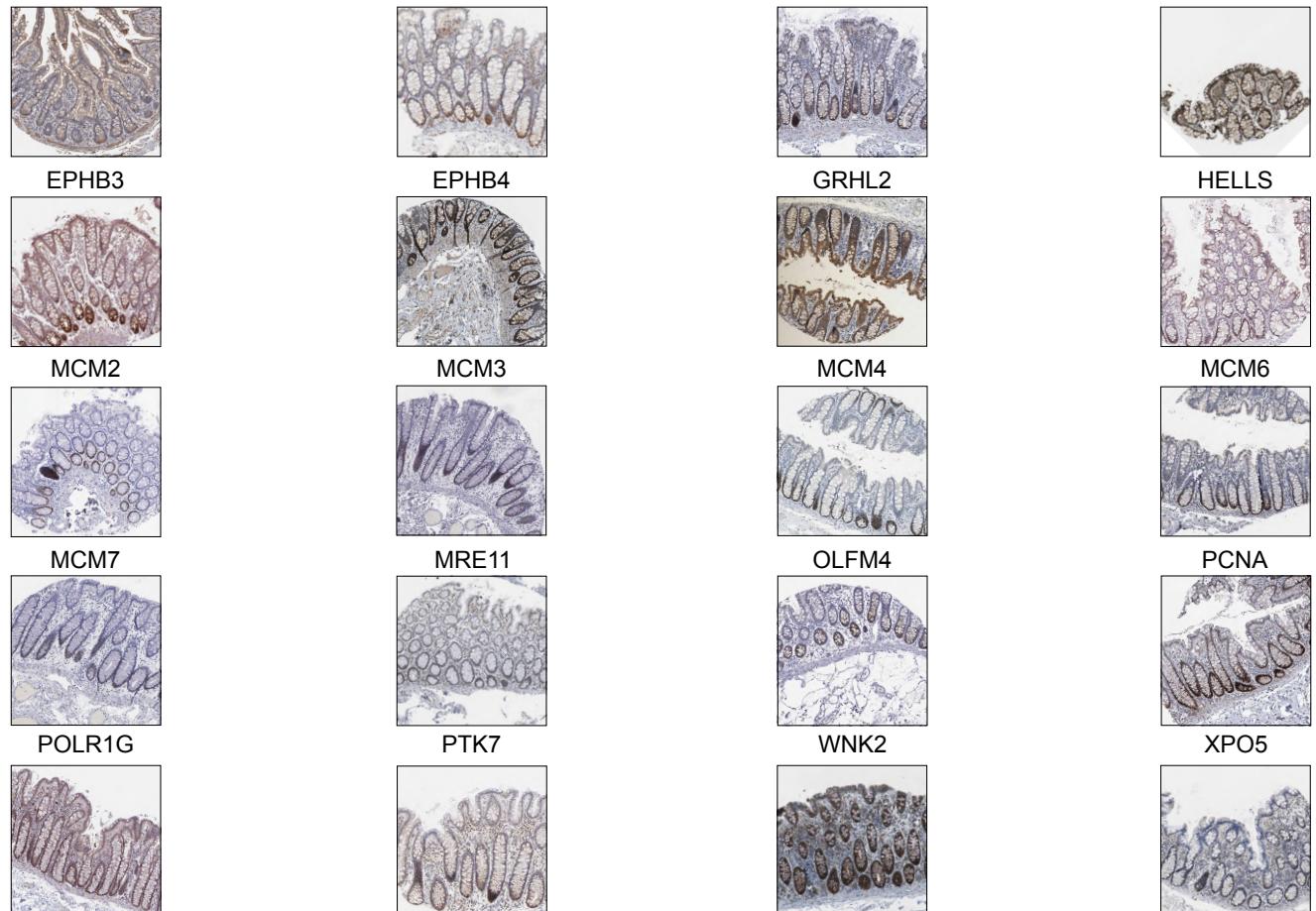
A**B****Figure S4**

Figure S4

A Spatial distribution of proteins that were identified in the colon stem cell signature. **B** Immunohistochemistry staining from the Human Protein Atlas⁴³ in human colon of proteins that were identified as potential colon stem cell markers.

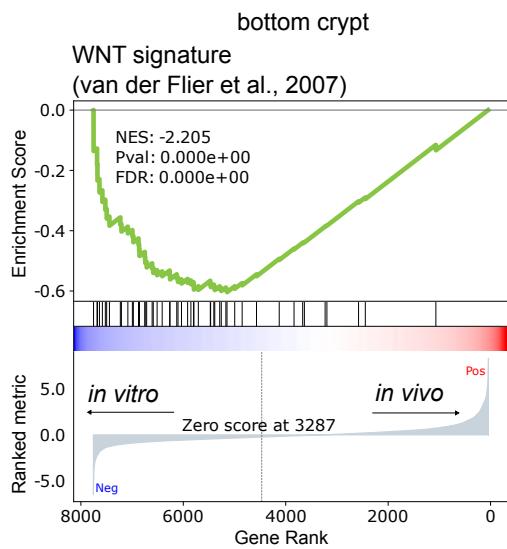
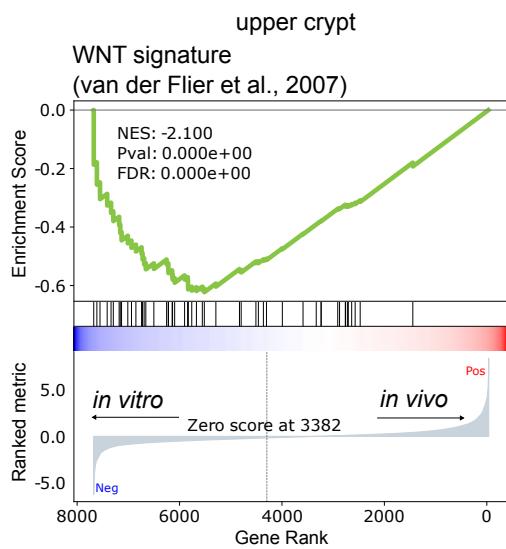
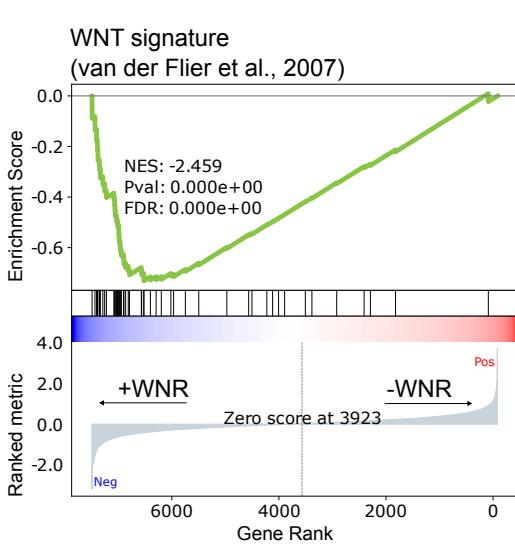
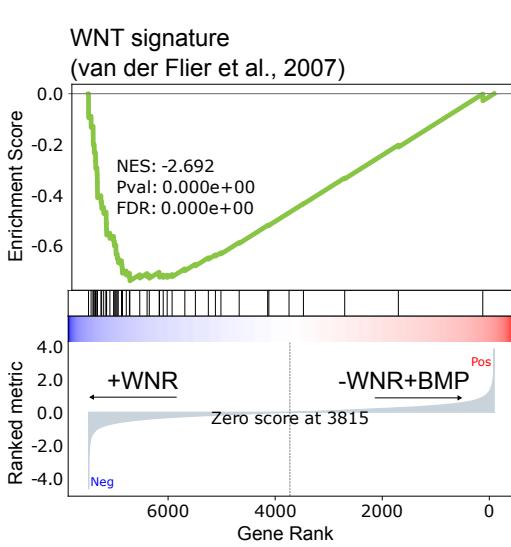
A**B****C****D**

Figure S5

Figure S5

A Gene set enrichment analysis of WNT signature proteins⁵¹ in crypt bottom IECs *in vivo* versus stem cells *in vitro*, **B** in upper crypt IECs *in vivo* versus LGR5-TdTomato⁺ cells *in vitro*, **C** organoids grown under -WNR versus +WNR conditions and **D** organoids grown under -WNR+BMP and +WNR conditions.

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