

1 **Single Cell Analysis Reveals Multi-faceted miR-375**

2 **Regulation of the Intestinal Crypt**

3

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46 **Summary**

47 The role of individual miRNAs in small intestinal (SI) epithelial homeostasis is under-explored.
48 In this study, we discovered that miR-375 is among the most enriched miRNAs in intestinal
49 crypts and stem cells (ISCs), especially facultative ISCs. We then showed by multiple
50 manipulations, including CRISPR/Cas9 editing, that miR-375 is strongly suppressed by Wnt-
51 signaling. Single-cell RNA-seq analysis of SI crypt-enriched cells from miR-375 knockout (375-
52 KO) mice revealed elevated numbers of tuft cells and increased expression of pro-proliferative
53 genes in ISCs. Accordingly, the genetic loss of miR-375 promoted resistance to helminth
54 infection and enhanced the regenerative response to irradiation. The conserved effects of miR-
55 375 were confirmed by gain-of-function studies in *Drosophila* midgut stem cells *in vivo*.
56 Moreover, functional experiments in enteroids uncovered a regulatory relationship between miR-
57 375 and Yap1 that controls cell survival. Finally, analysis of mouse model and clinical data
58 revealed an inverse association between miR-375 levels and intestinal tumor development.

59 60 **Highlights**

- 61 • miR-375 is one of the most enriched miRNAs in ISCs, especially facultative ISCs.
- 62 • miR-375 modifies tuft cell abundance and pro-proliferative gene expression in ISCs.
- 63 • Loss of miR-375 in mice enhances the host response to helminth infection and crypt
64 regeneration.
- 65 • Mouse and human intestinal cancer are associated with reduced miR-375 expression.

66 67 **eTOC Blurb**

68 Sethupathy and colleagues show that miR-375 is a Wnt-responsive, ISC-enriched miRNA that
69 serves as a brake on intestinal crypt proliferation. They also show that miR-375 modulates tuft

70 cell abundance and pro-proliferative gene expression in ISCs, that miR-375 loss enhances the
71 host response to helminth infection as well as crypt regeneration post-irradiation, and its reduced
72 expression is associated with intestinal cancer.

73

74 **Introduction**

75

76 The small intestinal epithelium is a highly proliferative and regenerative tissue compartment of
77 the intestinal mucosa. Actively-cycling intestinal stem cells (ISCs) located at the base of the
78 epithelial crypts drive homeostatic renewal (3-5 days) of the epithelial lining (Barker, 2014),
79 whereas facultative stem cells are induced upon injury to regenerate the epithelium (Ayyaz et al.,
80 2019; Richmond et al., 2016). ISCs give rise to several different specialized cell types, which
81 carry out diverse functions, including nutrient absorption and protection against pathogenic
82 infection. To ensure the integrity of the epithelial lining and maintain proper cell lineage
83 allocation, the proliferative capacity of actively-cycling ISCs, as well as injury-inducible
84 facultative ISCs, must be tightly regulated by various cell-autonomous signaling pathways
85 (Henning and von Furstenberg, 2016).

86

87 The Wnt signaling pathway is especially critical for determining ISC self-renewal and
88 proliferative capacity (He et al., 2004). In the stem cells of other organ systems, regulatory
89 RNAs (such as microRNAs) have been shown to play significant roles in modulating Wnt and
90 other related signal transduction pathways (Peng et al., 2016; Zhang et al., 2019). However, the
91 roles of most regulatory RNAs in the control of intestinal crypt behavior remain to be
92 investigated.

93

94 MicroRNAs (miRNAs) are small, ~22nt regulatory RNAs that serve as fine-tuners of gene
95 expression at the post-transcriptional level (Bartel, 2018; Gebert and MacRae, 2019). Their
96 activity is critical in a wide array of biological processes, including growth and differentiation
97 (Ivey and Srivastava, 2010). Over the past several years, it has been increasingly recognized that
98 miRNAs impact the structure and function of the small intestine. McKenna and colleagues
99 showed that gut-specific deletion of *Dicer1*, a critical processing enzyme of miRNAs, produces
100 profound effects on intestinal architecture and allocation of mature cell types (McKenna et al.,
101 2010). We previously reported that miRNAs in Sox9-Low jejunal epithelial cells, which are
102 partially enriched for ISCs, are highly sensitive to microbes (Peck et al., 2017a). We also
103 recently reported on a specific miRNA, miR-7, which is enriched along the enteroendocrine
104 lineage trajectory and regulates intestinal epithelial proliferation (Singh et al., 2020). Others
105 have shown that microRNAs such as miR-31, miR-34a, and miR-34b/c regulate mouse ISC
106 proliferation (Bu et al., 2016; Jiang and Hermeking, 2017; Tian et al., 2017). Moreover, the
107 evolutionary conserved role of microRNAs in regulating ISC activity is observed in the ability of
108 let-7, miR-305, and miR-263a to modulate ISC division in *Drosophila melanogaster* (Chen et
109 al., 2015; Foronda et al., 2014; Kim et al., 2017). Despite these advances, which miRNAs are
110 enriched in mouse actively-cycling or facultative ISCs, and if/how they alter the cellular
111 landscape and function of the intestinal crypt, remains unknown.

112

113 In this study, using several different reporter mice and fluorescence activated cell sorting (FACS)
114 methods, we identify miR-375 as the most enriched miRNA in crypts (and especially facultative
115 ISCs) relative to villus cells. Single cell RNA-seq reveals that genetic loss of miR-375 results in

116 the elevation of Wnt signaling within ISCs and an increase in the number of tuft cells.
117 Accordingly, we show that 375-KO mice exhibit significant reduction in worm burden after
118 *Heligmosomoides polygyrus* infection and in the regenerative response to whole-body
119 irradiation. The effects of miR-375 on intestinal epithelial survival and proliferation are further
120 confirmed by functional experiments in *Drosophila* midgut stem cells *in vivo* and murine
121 enteroids *ex vivo*, the latter of which also reveal a regulatory relationship between miR-375 and
122 *Yap1*. Finally, analysis of data generated from mouse models and human samples reveals an
123 association between miR-375 levels and intestinal tumor development.

124

125 **Results**

126

127 ***MiR-375 is the most enriched miRNA in intestinal stem cells***

128

129 ISCs, transit-amplifying progenitors, and cells that contribute to the stem cell niche are enriched
130 in intestinal crypts relative to villi (Barker, 2014). First, we profiled miRNAs, by small RNA
131 sequencing (small RNA-seq), in mouse jejunal crypts and villi (fractions validated by RT-qPCR,
132 **Fig. S1A,B**) separately and identified seven miRNAs that are significantly enriched in the crypts
133 (fold change > 2.5, P-value < 0.05) (**Fig. 1A, Table S1**). To determine the extent to which ISCs
134 contribute to the apparent crypt enrichment of these seven miRNAs, we performed small RNA-
135 seq on three different sorted populations of crypt-resident cells that have previously been shown
136 to exhibit stem cell features: the crypt-based columnar Lgr5-High cells (Barker et al., 2007)
137 (validated by RT-qPCR, **Fig. S1C**), Sox9-Low cells (Formeister et al., 2009) (validated by RT-
138 qPCR, **Fig. S1D**), and Cd24-Low cells (von Furstenberg et al., 2011). Bioinformatic analysis of

139 the small RNA-seq data revealed that 14 miRNAs are shared among the top 20 most highly
140 expressed miRNAs in each of the three cell populations (**Fig. 1B, Table S2**). Among these 14,
141 only miR-375 is also among the seven crypt-enriched miRNAs (**Fig. 1A, Table S1**), which we
142 further validated by quantitative real time qPCR (RT-qPCR) (**Fig. 1C**). Moreover, miR-375 is
143 the most highly enriched miRNA in crypt-resident Lgr5-High ISCs compared to cells over-
144 represented in villi (Sox9-Neg) (**Fig. 1D**). It is also the most highly enriched in crypt-resident
145 Sox9-Low ISCs relative to Sox9-Neg (**Fig. 1E**). Other crypt-based cells include slowly-cycling
146 Lgr5-negative facultative ISCs, (lower side population; LSP) (Dekaney et al., 2005) (validated
147 by RT-qPCR, **Fig. S1E**). Although these cells are more slowly cycling than Lgr5+ ISCs at
148 baseline, they have the capacity to revert to a more proliferative state in response to injury
149 (Ayyaz et al., 2019; Richmond et al., 2016). We sorted and sequenced LSP cells and found that
150 miR-375 is even more enriched in this population relative to Lgr5-High ISCs and upper side
151 population (USP) actively-cycling stem cells (**Fig. 1F**), respectively. Taken together, these data
152 indicate that miR-375 is over-represented in crypts, expressed in actively-cycling ISCs, and even
153 more highly expressed in facultative ISCs.

154

155 *miR-375 expression is responsive to Wnt signaling in intestinal epithelial cells*

156

157 Wnt signaling plays a prominent role in maintaining ISC survival and proliferative capacity
158 (Flanagan et al., 2018). The high expression of miR-375 in Lgr5+ ISCs suggests that either miR-
159 375 promotes or serves as a break on Wnt signaling pathways. Because miR-375 expression is
160 even higher in facultative ISCs (**Fig. 1F**), which exhibit reduced Wnt signaling activity relative

161 to Lgr5⁺ ISCs (Tao et al., 2015), we hypothesize that miR-375 and Wnt signaling are mutually
162 suppressive.

163

164 To determine whether miR-375 is suppressed by Wnt signaling, we assessed miR-375 expression
165 under various conditions in which Wnt signaling is perturbed. First, we considered the
166 physiological condition of aging in which Wnt signaling in ISCs is reduced (Nalapareddy et al.,
167 2017). Comparing expression in intestinal crypts of >1-year old mice relative to those derived
168 from mice 5 months or younger, we observed a nearly 2-fold increase in mature miR-375 levels
169 (**Fig. 1G**). Second, exposure of mouse jejunal enteroids to 1 μ M IWP2, a porcupine inhibitor
170 and Wnt antagonist (Mo et al., 2013), significantly increased expression of miR-375 relative to
171 the mock condition (**Fig. 1H**). Finally, we used CRISPR/Cas9 to generate enteroids with a Wnt-
172 activating mutation in APC (APCmut) (Dow et al., 2015) and performed small RNA sequencing
173 analysis. We found that miR-375 is among the few miRNAs that are significantly suppressed in
174 APCmut enteroids relative to unmutated control enteroid cells (**Fig. 1I, Table S3**). This finding
175 was validated by RT-qPCR in APCmut enteroids as well as enteroids with a Wnt-activating β -
176 catenin mutation (β -Cat-mut), also generated by CRISPR/Cas9 (**Fig. 1J**). Taken together, the
177 results of these analyses indicate that miR-375 expression is controlled by the Wnt signaling
178 pathway.

179

180 *miR-375 deficiency enhances small intestinal tuft cell abundance and enhances resistance to*
181 *Heligmosomoides polygyrus helminth infection*

182

183 The enrichment of miR-375 expression in intestinal stem cells and its responsiveness to Wnt
184 signaling suggested that miR-375 may play a role in affecting the allocation and/or proliferation
185 of various intestinal crypt cell types. To test this, we performed single cell RNA-seq (scRNA-
186 seq) on jejunal crypt cells from 12-month-old WT mice and mice with a germ-line deletion for
187 miR-375 (375-KO) (see Methods & Materials). Whole-body disruption of the miR-375 gene in
188 375-KO mice relative to WT was confirmed by tail genomic DNA genotyping (**Fig. S2A**) and by
189 RT-qPCR data of miR-375 expression in jejunal enteroids derived from 375-KO mice relative to
190 WT (**Fig. S2B**). In our analysis of the scRNA-seq data, we were able to identify several distinct
191 clusters of cell populations (**Fig. 2A**) and unambiguously identify each of them as a well-
192 established intestinal epithelial cell type based on the expression of distinguishing marker genes
193 (**Fig. 2B**) as defined in a seminal scRNA-seq study of intestinal crypts by Haber and coworkers
194 (Haber et al., 2017). In terms of the relative abundance of different cell types, the most striking
195 result was a robust increase in tuft cells in 375-KO compared to WT (**Fig. 2C**). Gene expression
196 analysis confirmed that several tuft cell marker genes including *Pou2f3*, *Gfi1b*, *Ascl2*, *Dclk1*,
197 *Trpm5*, and *Rgs13* are elevated in 375-KO mice relative to WT (**Fig. 2D**). One of the best-
198 characterized functions of intestinal tuft cells is to mediate the host anti-helminth response
199 (Gerbe et al., 2016). Therefore, we hypothesized that the loss of miR-375 may reduce infectious
200 burden. To test this hypothesis, both WT and 375-KO mice were infected with the helminth
201 *Heligmosomoides polygyrus*. After 14 days of inoculation, although 375-KO mice did not
202 display increased numbers of total immune cells (**Fig. S3A**) within the mesenteric lymph node,
203 they did exhibit significantly increased numbers of eosinophils (**Fig. 2E**), which is a well-
204 established marker of a robust host response to helminth infection. Consistent with this result, we

205 found that 375-KO mice exhibited a significant decrease in intestinal worm burden (**Fig. 2F**),
206 indicative of enhanced resistance to helminth infection.

207

208 *miR-375 deficiency enhances Wnt signaling in intestinal stem cells and promotes the*
209 *regenerative response to irradiation*

210

211 Besides elevating tuft cell abundance, the scRNA-seq analysis of 375-KO and WT mice
212 indicated that loss of miR-375 alters pathways in ISCs as well. Network analysis of the genes
213 up-regulated in ISCs in 375-KO vs. WT showed enrichment of several transcription factors
214 associated with the Wnt signaling pathway (**Fig. 3A**). Consistent with this result, we found that
215 Wnt signaling target genes, including *Ccnd2*, *Cd44*, *Ascl2*, *Myc*, *Cldn2*, and *Trim65* are
216 significantly up-regulated in ISCs from 375-KO mice compared to WT (**Fig. 3B,C**). These data
217 suggested that miR-375 deficiency promotes Wnt signaling, which may elevate the proliferative
218 capacity of the intestinal epithelium. The loss of miR-375 did not result in alteration of either
219 mid-jejunal crypt depth (**Fig. S4A,B**) or the number of proliferating PH3⁺ mid-jejunal crypt cells
220 (**Fig. S4C,D**) under unchallenged, baseline conditions. We hypothesized that the effect of miR-
221 375 loss may be more pronounced in response to injury. To test this hypothesis, we evaluated
222 the intestinal crypt response to whole-body irradiation in 375-KO mice compared to WT. Initial
223 assessment of changes in mid-jejunal crypt depth of WT mice suggested that whole-body
224 irradiation of 10 Gy results in peak crypt regeneration around 3 days post-irradiation (**Fig. S5A**).
225 Therefore, we evaluated mid-jejunal crypt depth of WT and 375-KO mice during a period of
226 increasing crypt regeneration at 1 day and 2.5 days post-irradiation (p.i.). Accelerated deepening
227 of 375-KO mid-jejunal crypts in response to irradiation relative to WT indicated that reduced

228 miR-375 expression potentiates intestinal regeneration induced by radiation injury (**Fig. 3D,E**).
229 To determine whether other external challenges produce a similar phenotype, we subjected WT
230 and 375-KO mice to a high fat diet for 16 weeks. There was no significant difference in weight
231 gain between WT and 375-KO mice (**Fig. S4E**). Furthermore, both groups of mice manifested
232 similar mid-jejunal crypt depths (**Fig. S4F,G**), indicating that the functional relevance of miR-
233 375 to the regulation of crypt depth is specific to the regenerative response to irradiation and not
234 generalizable to any stress condition.

235

236 *Loss of miR-375 leads to increased intestinal epithelial proliferation*

237

238 We next sought to assess the function of miR-375 considering the intestinal epithelium alone.
239 Specifically, we isolated jejunal crypts from 375-KO and WT mice and established enteroids *ex*
240 *vivo*. The enteroids from 375-KO mice exhibited a significantly greater budding index relative
241 to WT (**Fig. 4A,B**). 375-KO jejunal enteroids also displayed elevated survival compared with
242 WT jejunal enteroids, an effect that was abrogated by inhibition of Wnt signaling (**Fig. 4C,D**).

243

244 We repeated the *ex vivo* study using enteroids from WT mice treated with either a locked nucleic
245 acid (LNA) inhibitor of miR-375 (LNA-375) or LNA-scramble control (LNA-scr). Treatment
246 with LNA-375 led to a ~1,000-fold loss in the levels of miR-375 (**Fig. S6A**). Though loss of
247 miR-375 did not affect enteroid size (**Fig. S6B,C**), it did lead to significantly greater budding
248 (**Fig. 4E,F**) and proliferation as measured by PH3+ cells/enteroid (**Fig. 4G,H**).

249

250 Given the extensive conservation of miR-375 across many species (**Fig. 5A**), we next evaluated
251 the effect of miR-375 overexpression *in vivo* in the midgut epithelium of *Drosophila*
252 *melanogaster*, a well-established model for gut stem cell response to stress and
253 infection (Buchon et al., 2009). First, we performed overexpression of miR-375 using the
254 lineage tracing system *esg^{F/O}*. In this system, GFP and miR-375 are both expressed in *esg*⁺ ISCs
255 and progenitor cells, as well as all direct progeny. We found that miR-375 reduces the
256 generation of new GFP⁺ cells (**Fig. 5B**), suggesting that it suppresses ISC/progenitor cell
257 activity. We next performed over-expression of miR-375 using *esg^{TS}* (*esgGal4, Gal80^{TS}>UAS-*
258 *miR-375*), in which GFP and miR-375 are expressed only in *esg*⁺ ISCs and progenitor cells. We
259 found that miR-375 did not alter the number of GFP⁺ ISCs and progenitor cells in basal
260 homeostatic conditions (**Fig. 5C**). However, midguts under these basal conditions have a limited
261 amount of proliferative activity; therefore, we repeated the experiment of miR-375 over-
262 expression in ISCs and progenitor cells (*esgGal4, Gal80^{TS}>UAS-miR-375*) under stress
263 conditions of infection with *Erwinia carotovora carotovora* 15 (*Ecc15*), a fly pathogen that
264 induces high levels of proliferation (Buchon et al., 2010). We found that miR-375 robustly
265 decreases the number of GFP⁺ cells (**Fig. 5D**), suggestive of reduced cycling. Consistent with
266 this finding, we also showed that over-expression of miR-375 in ISCs and progenitor cells led to
267 a significant reduction in the total number of PH3⁺ proliferating cells (**Fig. 5E**). These results
268 demonstrate that miR-375 is a strong cell-autonomous regulator of ISC proliferation and attest to
269 the broad species conservation of the effect of miR-375 on intestinal epithelial proliferation.
270
271 In full, these data demonstrate that both genetic deletion (permanent) and pharmacologic
272 suppression (transient) of miR-375 promotes intestinal epithelial growth phenotypes in murine

273 enteroids *ex vivo* and suggest a function for miR-375 in controlling stress-induced proliferation
274 in the *Drosophila* midgut *in vivo*.

275

276 ***miR-375 control of enteroid growth is mediated in part by regulation of the Yap1 pathway***

277

278 To identify candidate mechanisms by which miR-375 exerts its effects on intestinal epithelial
279 proliferation, we mined our previously published small RNA-seq and RNA-seq data in Sox9-
280 Low stem cells under diverse conditions (chow diet, high-fat diet, germ-free, and microbially-
281 conventionalized) that variably influence the proliferative state of intestinal crypts (Beyaz et al.,
282 2016; Peck et al., 2017a). Analysis of these data revealed that the expression of miR-375 and the
283 Hippo signaling pathway factor *Yap1* (Yu et al., 2015), a target of miR-375 (Liu et al., 2010), are
284 very strongly inversely correlated (Pearson's $r = -0.808$) (**Fig. 6A**). We then treated enteroids
285 established from 375-KO and WT mice with either 2 μM or 5 μM Yap1 inhibitor (verteporfin)
286 (Liu-Chittenden et al., 2012) and compared to the untreated condition (mock). As expected,
287 verteporfin exerted a dose-dependent suppressive effect (mock vs. 2 μM vs. 3 μM) on the Yap1
288 target gene *Ctgf* (**Fig. S7A**) (Huntoon et al., 2010). Budding efficiency did not appear to be
289 affected by Yap1 inhibition (**Fig. 6B,C**). However, Yap1 inhibition did reduce enteroid survival
290 and, notably, this effect at 2 μM verteporfin was almost completely rescued by the loss of miR-
291 375, but not at 5 μM verteporfin (**Fig. 6D,E**). In effect, miR-375 deficiency compensates for the
292 anti-survival effect of moderate, but not strong, inhibition of Yap1.

293

294 ***Intestinal tumors are strongly associated with diminished miR-375 expression***

295

296 Elevated Wnt (Schneikert and Behrens, 2006) and Yap1 signaling (Zhao et al., 2011) are both
297 associated with the development of intestinal cancers. Therefore, we hypothesized that the
298 development of intestinal tumors may be associated with reduced expression of miR-375. Small
299 intestinal polyps collected from mice with Wnt activating mutations of APC (APCmut),
300 including APCmin and APCq1405x mutations, exhibit decreased levels of miR-375 relative to
301 WT mice (**Fig. S8A**). To determine whether human colon tumors show a similar association, we
302 interrogated the The Cancer Genome Atlas (TCGA) database to compare microRNA levels in
303 primary colon adenocarcinoma tumors to non-tumor colon samples. We found that miR-375 was
304 the most highly expressed miRNA in non-tumor colon tissue that is significantly downregulated
305 (>5-fold) in tumor relative to non-tumor tissue (**Fig. 7A,B**). This pattern of miR-375 expression
306 is preserved when comparing only the matched tumor and non-tumor samples from the same
307 individual (**Fig. 7C**). Notably, integrative analysis with TCGA RNA-seq data revealed that,
308 among miR-375 target genes down-regulated in colon adenocarcinoma samples, *YAP1* is the
309 most inversely correlated gene with miR-375 expression (**Fig. 7D**).

310

311 Taken together, the data presented in this study, which features crypt scRNA-seq, as well as
312 cross-species functional studies, provide a comprehensive view of the role of a miRNA in the
313 intestinal crypt. Important functions of miR-375 revealed in this work include the control of tuft
314 cell abundance and response to helminth *Heligmosomoides polygyrus* infection as well as Wnt-
315 signaling in ISCs and regenerative response to irradiation. A working model of miR-375
316 expression and function is shown in **Fig. 7E**.

317

318 **Discussion**

319

320 It is recognized that miRNAs affect the structural and functional properties of the small intestinal
321 epithelium (McKenna et al., 2010; Peck et al., 2017a; Singh et al., 2020). Previously, we have
322 shown that an intestinal epithelial cell population that is partially enriched for ISCs is also
323 enriched for several miRNAs that are responsive to microbes (Peck et al., 2017a). In this report,
324 we have: (1) identified 14 miRNAs that are very highly expressed in mouse jejunal ISCs
325 including the most enriched miRNA miR-375; (2) shown that the expression of miR-375 is
326 strongly suppressed by Wnt signaling; (3) found that genetic loss of miR-375 results in an
327 elevation of tuft cell numbers and in the significant reduction of helminth worm burden after
328 *Heligmosomoides polygyrus* infection; (4) determined that miR-375 deficiency promotes the
329 Wnt-signaling pathway in ISCs and enhances the intestinal epithelial regenerative response to
330 irradiation injury; (5) demonstrated that miR-375 regulates intestinal epithelial survival and
331 growth in murine enteroids *ex vivo* and *Drosophila* midgut *in vivo*; (6) shown that the loss of
332 miR-375 can partially compensate for the anti-survival effect of Yap1 inhibition in murine
333 enteroids; and (7) revealed that diminished expression of miR-375 is associated with intestinal
334 tumor development in both mouse and human.

335

336 To our knowledge, this is the first report of the profiling of miRNA expression in Lgr5-High
337 ISCs. Additionally, although previous reports from us and others have described the importance
338 of miRNAs in the enteroendocrine cell lineage trajectory (Knudsen et al., 2015; Singh et al.,
339 2020), this study is the first to our knowledge to investigate the function of an ISC-enriched
340 miRNA. Furthermore, this is also the first study to our knowledge that employs single cell
341 RNA-seq to investigate intestinal cell type-specific changes in gene expression in the genetic

342 absence of a miRNA. We found that at baseline the loss of miR-375 promotes tuft cell numbers,
343 and accordingly, enhances eosinophil abundance and reduces *Heligmosomoides polygyrus*
344 burden after infection. The detailed underlying molecular mechanisms merit further
345 investigation. Also, we showed that the loss of miR-375 promotes the intestinal epithelial
346 regenerative response to irradiation. Follow-up functional *ex vivo* studies in enteroids and *in*
347 *vivo* studies in *Drosophila* midgut revealed that miR-375 contributes to the control of intestinal
348 epithelial cell survival and proliferation, in part by regulation of Yap1. Likely, though, miR-375
349 has many molecular targets beyond Yap1, and their identification warrant further study in the
350 future.

351

352 Luminal changes in microbes and nutrients can also greatly influence the way ISCs effect
353 changes in intestinal epithelial proliferation (Biton et al., 2018; Mah et al., 2014; Peck et al.,
354 2017b). Our earlier report demonstrated that miR-375 is enriched in Sox9-Low cells, which are
355 partially enriched for ISCs, and that its expression is markedly downregulated with exposure to
356 luminal microbes (Peck et al., 2017a). This dependence may mediate the intestinal proliferative
357 changes that are seen during postnatal intestinal development (Al-Nafussi and Wright, 1982) or
358 with exposure to certain antibiotics (Hormann et al., 2014) or certain infectious agents (Santos et
359 al., 2016). In addition to microbial influences, miR-375 has been associated with responding to
360 host metabolic changes. Besides helping to determine the enteroendocrine lineage (Knudsen et
361 al., 2015), it has a well characterized role in regulating secretion of insulin from pancreatic β -
362 cells in response to glucose (Eliasson, 2017). Provision of a chronic high fat diet has been
363 observed to enhance ISC number and the proliferation of the intestinal epithelium (Mah et al.,
364 2014). However, our current examination of the effect of genetic deletion of miR-375 on ISC

365 activity in response to high fat diet has not revealed any significant changes in intestinal
366 epithelial growth properties *in vivo*.

367

368 Finally, agents that damage ISCs, such as irradiation and chemotherapies, and that cause acute
369 intestinal injury, will lead to enhanced intestinal proliferation after exposure (Dekaney et al.,
370 2009; Gurley et al., 2017). Stem-like IECs that have lineage determining multipotency, such as
371 those IECs that are enriched in Lower Side Population (LSP) FACS cells, can be induced under
372 these conditions to assume the function of actively cycling ISCs to repopulate and renew the
373 damaged intestinal epithelium (Roche et al., 2015; Yan et al., 2017). It is worth noting that in
374 this report we have found that not only is miR-375 enriched in ISCs relative to enterocytes, it is
375 further enriched in LSP cells relative to Lgr5-High ISCs. It is possible that the elevated
376 expression of miR-375 in these facultative stem cells may help preserve their reserve status by
377 setting a higher threshold for Wnt activation. They may be responsible for the enhancement of
378 intestinal epithelial regeneration that we observe in 375-KO mice in response to radiation injury.
379 Moreover, they may also contribute to the potential Wnt signaling-driven reduction in miR-375
380 expression that we observe associated with intestinal tumors. Altogether, our data concerning
381 miR-375's effect on ISC activity does give some indications that miR-375 may be involved in
382 multiple aspects of physiological and pathophysiological regulation of ISCs that merit further
383 detailed investigation in the future.

384

385 **Experimental Procedures**

386

387 **Mouse models.** The following mice were utilized: female and male Sox9-EGFP (Formeister et
388 al., 2009), female Lgr5-EGFP (Sato et al., 2009), female and male wild-type C57BL/6, male
389 wild-type and miR-375 null B62J (albino C57BL6/2J), male APC^{min}, and male APC^{q1405x}.
390 The harvested small intestine was measured and divided into three equal segments. The middle
391 region was considered jejunum. All animal procedures were performed with the approval and
392 authorization of the Institutional Animal Care and Use Committee at each participating
393 institution. Mice were used in these experiments due to their tractability to genetic manipulation,
394 including deletion of the microRNA of interest, as well as the availability of a wide array of
395 appropriate experimental reagents. Mice were housed in well-ventilated cages under 12 hr
396 light/dark cycles with free access to water and standard chow in addition to tubing for
397 environmental enrichment. During experimentation, the mice were monitored at regular
398 intervals to determine their well-being, and at the time of tissue collection the mice were
399 anesthetized by CO₂ inhalation and euthanized by means of cervical dislocation.

400
401 **Generation of miR-375 knockout mice using CRISPR/Cas9.** To delete the miR-375 gene in the
402 mouse, we used the CRISPR/Cas9 system with a guide RNA (gRNA) targeting the 946-965 bp
403 region of the mouse miR-375 gene (ENSMUSG00000065616). 173 FVBxB62J F1 hybrid 1-cell
404 embryos were co-injected with 2.5 µg of gRNA and 7.5 µg of Cas9 mRNA. 107 2-cell embryos
405 were transferred to pseudo-pregnant recipient female mice at ~20 embryos/recipient. 20 founder
406 pups were born and validated for deletion of the miR-375 gene by targeted genomic sequencing.
407 Mice null for miR-375 were backcrossed at least three generations with B62J albino wildtype
408 mice. Synthesis of the gRNA and mouse embryo co-injections and viable embryo emplacement

409 were performed by the Cornell Stem Cell and Transgenic Core Facility at Cornell University
410 (work supported in part by Empire State Stem Cell Fund, contract number C024174).

411

412 ***Mouse helminth infection.*** The lifecycle of *Heligmosomoides polygyrus* was maintained in
413 C57BL/6 mice as previously described (Camberis et al., 2003; Johnston et al., 2015). WT and
414 375-KO B62J mice were infected by oral gavage with 200 *Heligmosomoides polygyrus* L3
415 larvae. On day 14 post infection, adult worm burdens were assessed by counting the number of
416 worms located in the entire length of the small intestine following exposure of the lumen by
417 dissection.

418

419 ***Immune cell isolation.*** For assessment of Type 2 immune cell responses in the intestine, the
420 murine mesenteric lymph nodes (MLNs) were harvested. Single cell suspensions of murine
421 MLNs were prepared by mashing MLNs through a 70 μm cell strainer and counting total number
422 of cells.

423

424 ***Whole-body mouse irradiation.*** 4-5 months old male WT and 375-KO B62J mice were
425 subjected to 10 Gy of whole-body X-radiation in a cesium-137 irradiator with rotating turntable.

426

427 ***Mouse high fat diet study.*** 2 months old male WT and 375-KO B62J mice were fed a high fat
428 diet (45% kcal provided by fat) (D12451, Research Diets Inc., New Brunswick, NJ) ad libitum
429 for 14-15 weeks. Body weights were measured weekly.

430

431 ***Mouse small intestinal villi and polyp isolation.*** Small intestinal villi were collected from
432 C57BL/6 mice by scraping the mucosal surface of cold PBS-flushed and longitudinally cut
433 whole small intestinal tissue with a glass coverslip. Isolated villi were pelleted by centrifugation
434 (110 x g for 5 minutes at 4°C) and flash frozen. Using a dissection microscope, small intestinal
435 polyps were identified and individually collected from cold PBS-flushed and longitudinally cut
436 small intestine from APC^{min} and APC^{q1405x} mice. Polyps were subsequently flash frozen.

437
438 ***Flow cytometry.*** Four distinct mouse-based cell marker systems were used to sort ISCs (Sox9-
439 EGFP; Lgr5-EGFP; and Cd24) and stem-like IECs (Side Population). Mouse intestinal epithelial
440 cells from the jejunum were dissociated and prepared for fluorescence-activated cell sorting
441 (FACS) as described previously (Mah et al., 2014). For Sox9-EGFP, Lgr5-EGFP, and Side
442 Population cell sorts: CD31-APC (BioLegend, San Diego, CA, cat. 102416), CD45-APC
443 (BioLegend, cat. 1032124), Annexin-V-APC (Life Technologies, Carlsbad, CA, cat. A35110),
444 and Sytox-Blue (Life Technologies, cat. S34857) staining were used to exclude endothelial cells,
445 immune cells, apoptotic cells, and nonviable cells, respectively. The gating parameters of FACS
446 sorting were described previously (Mah et al., 2014). For Cd24-Low cell sorts, UEA-FITC
447 (Vector Laboratories, Burlingame, CA, cat. FL-1061), CD45-FITC (BioLegend, San Diego, CA,
448 cat. 553080), and propidium iodide (BioLegend, cat. 421301) staining was used to exclude
449 goblet/Paneth, endothelial, and nonviable cells, respectively. In addition, for these sorts CD24-
450 Pac Blue (BioLegend, cat. 101819) and EpCAM-PECy7 (BioLegend, cat. 118215) staining was
451 used to positively select for CD24⁺ epithelial cells. The Sox9, Lgr5, and Side Population sorts
452 were performed using a Mo-Flo XDP cell sorter (Beckman-Coulter, Fullerton, CA) at the
453 University of North Carolina Flow Cytometry Core Facility. Sorting of Cd24-Low cells was

454 conducted at North Carolina State University, College of Veterinary Medicine using a Mo-Flo
455 XDP cell sorter (Beckman-Coulter, Fullerton, CA). The cells were sorted directly into cold
456 DMEM or lysis buffer.

457

458 Side population sorting was used to separate the sub-fraction of slowly cycling from active
459 cycling intestinal stem cells, as described previously (von Furstenberg et al., 2014). Mouse
460 intestinal epithelial cells from the jejunum of female C57BL/6 mice were prepared and sorted
461 into either upper side population (consisting of actively cycling stem cells) or lower side
462 population (consisting of slowly cycling stem cells) by the previously described gating methods
463 (von Furstenberg et al., 2014). The side population sorting was performed using a Mo-Flo XDP
464 cell sorter (Beckman-Coulter, Fullerton, CA) at the University of North Carolina Flow
465 Cytometry Core Facility. Cells were sorted directly into cold lysis buffer (Norgen Biotek,
466 Thorold, ON, Canada).

467

468 Immune cell suspensions from helminth-infected mice were incubated with Aqua Live/Dead
469 Fixable Dye (Life Technologies, Grand Island, NY) and fluorochrome-conjugated monoclonal
470 antibodies (mAbs) against mouse CD3 (17A2), CD4 (GK1.5), CD5 (53-7.3), CD11b (M1/70),
471 CD11c (N418), CD19 (eBio1D3), CD25 (PC61.5), CD45 (30-F11), CD45.1 (A20), CD45.2
472 (104), CD127 (eBioSB/199), CD90.2 (53-2.1), IL-33R (RMST2-2), IL-25R (MUNC33), NK1.1
473 (PK136), Gata3 (TWAJ) or Siglec-F (E50-2440, BD Biosciences, San Jose, CA). All antibodies
474 from Thermo Fisher unless otherwise noted. Eosinophils, ILC2s and CD4 T helper 2 cells (Th2)
475 were gated as live, CD45⁺SiglecF⁺CD11b⁺; live, CD45⁺lin⁻CD90⁺CD127⁺ST2⁺CD4⁻ and live,
476 CD45⁺lin⁺CD90⁺CD4⁺Gata3⁺, respectively

477

478 ***Histological analysis.*** Mouse mid-jejunal tissue was fixed in 4% (v/v) neutral-buffered
479 paraformaldehyde, embedded in paraffin, and cut into 5 μm sections for various staining
480 experiments. Haemotoxylin and eosin (H&E) staining was performed for morphometric
481 analyses (crypt depth). Immunofluorescent staining of PH3 was performed to visualize
482 proliferating cells. Briefly, sections were incubated with primary antibody (rabbit anti-PH3,
483 1:100 dilution in immunofluorescence buffer,) (Cell Signaling, Danvers, MA, 9701S) overnight
484 at 4° C followed by goat anti-rabbit Alexa fluor 594 secondary antibody (1:400, Invitrogen,
485 Carlsbad, CA, cat. A1102) incubation for 1 hr at room temperature. Hoechst 33342 (1:1000,
486 Invitrogen, cat. C10637) was used to visualize nuclei. Images were captured using a BX53
487 Olympus scope (Olympus, Center Valley, PA).

488

489 ***RNA extraction and real-time qPCR.*** Total RNA was isolated using the Total or Single-cell
490 RNA Purification kit (Norgen Biotek, Thorold, ON, Canada). High Capacity RNA to cDNA kit
491 (Life Technologies, Grand Island, NY) was used for reverse transcription of RNA. TaqMan
492 microRNA Reverse Transcription kit (Life Technologies) was used for reverse transcription of
493 miRNA. Both miRNA and gene expression qPCR were performed using TaqMan assays (Life
494 Technologies) with either TaqMan Universal PCR Master Mix (miRNA qPCR) or TaqMan Gene
495 Expression Master Mix (mRNA qPCR) per the manufacturer's protocol on a BioRad CFX96
496 Touch Real Time PCR Detection System (Bio-Rad Laboratories, Richmond, CA). Reactions
497 were performed in triplicate using either U6 (miRNA qPCR) or Rps9 (mouse mRNA qPCR) as
498 the normalizer.

499

500 ***Small RNA library preparation and sequencing.*** The small RNA sequencing of cells from the
501 various cell sorts and from enteroids was conducted at Genome Sequencing Facility of Greehey
502 Children's Cancer Research Institute at University of Texas Health Science Center at San
503 Antonio. Libraries were prepared using the TriLink CleanTag Small RNA Ligation kit (TriLink
504 Biotechnologies, San Diego, CA). Seven to eight libraries were sequenced per lane with single-
505 end 50x on the HiSeq2500 platform. Raw sequencing data is available through GEO accession
506 GSE151088.

507

508 ***RNA library preparation and sequencing.*** RNA-sequencing libraries from the Sox9⁺-EGFP
509 sorts of chow-fed, high fat diet-fed, conventionalized, and germfree C57BL/6J mice were
510 prepared using the Clontech SMARTer Ultra Low Input library preparation kit combined with
511 Nextera XT DNA sample preparation kit (Illumina) and sequenced with single-end 100 bp on a
512 HiSeq2000 platform at the UNC High Throughput Sequencing Core Facility, as previously
513 described (Peck et al., 2017a). Raw sequencing data is available through GEO accession
514 GSE151088.

515

516 ***scRNA-seq library preparation and sequencing.*** Mouse jejunal crypts from 1 year old male WT
517 and 375-KO B62J mice were isolated as previously described (Mah et al., 2014; Peck et al.,
518 2017a). Isolated crypts were resuspended in an ice cold solution of PBS with 0.04% (w/v)
519 bovine serum albumin, and pelleted at 1000 x g at 4°C for 5 minutes. The crypts were
520 subsequently digested with 0.3U/mL dispase I in HBSS at 37°C for 12 minutes with gentle
521 agitation. After stopping dispase I activity with an addition of fetal bovine serum to a final
522 concentration of 10% (v/v), the single cell suspension was filtered, pelleted at 500 x g at 4°C for

523 5 minutes, washed with cold HBSS, filtered again, and then resuspended in an ice cold solution
524 of PBS with 0.04% (w/v) bovine serum albumin. Prior to submission, single cell suspensions
525 were triturated by pipetting and evaluated for total viable cell number by using trypan blue
526 staining with a TC20 automated cell counter (Bio-Rad Laboratories, Richmond, CA). Single cell
527 RNA sequencing of these samples was performed at the Cornell University Biotechnology
528 Resource Center. Libraries were prepared using the 10X Genomics Chromium preparation kit
529 (10X Genomics, Pleasanton, CA). Raw sequencing data is available through GEO accession
530 GSE151088.

531

532 ***Bioinformatics analysis.*** Small RNA-sequencing reads were aligned to the mouse genome
533 (mm9) and quantified using miRquant 2.0 as previously described (Kanke et al., 2016), with the
534 exception that raw miRNA counts were normalized using either RPM or DESeq2 (Love et
535 al., 2014) to determine significance. miRNA annotation was performed using miRbase (r18 for
536 mouse). RNA-sequencing reads were mapped to mouse genome release mm10 using STAR
537 (v2.5.3a) (Dobin et al., 2013) and transcript quantification was performed using Salmon
538 (v0.6.0) (Patro et al., 2017). Differential gene expression analysis was accomplished using
539 DESeq2 (Love et al., 2014). Single cell RNA-sequencing was performed using 10x genomics
540 cellranger software (v3.0.1) and aligned to the mouse genome (mm10) to get a cell count matrix.
541 Single cell RNA-seq data was subsequently filtered, clustered, visualized, and analyzed using
542 Seurat (v3.1.5). Clusters were assigned cell types using markers reported by Haber and
543 coworkers (Haber et al., 2017), and unassigned or immune-like clusters were discarded, resulting
544 in 2614 WT and 3080 375-KO B62J mouse cells.

545

546 ***TCGA analysis.*** Data Download: RNA-seq data from 382 primary colon tumor samples and 39
547 solid normal tissue were downloaded from the TCGA database. High Throughput Sequencing
548 (HTSeq) counts data were downloaded using the NIHGDC Data Transfer Tool and normalized
549 using DESeq2. miRNA data from 371 primary colon tumor samples and 8 solid normal tissue
550 were downloaded from the TCGA database. miRNA quantification data using mirbase21 were
551 downloaded using the NIHGDC Data Transfer Tool. Scatterplot graph to narrow down miR-
552 375:miRNAs were filtered for those that had an average expression above 50 RPMMM in the
553 solid normal tissue (n=8). Fold-change was calculated for each miRNA by adding 0.1 RPMMM
554 to all values and calculating the average expression for all tumor samples (n=371) and dividing
555 by the average for all solid normal tissue (n=8). A log₂ normalization was then applied to these
556 fold-change values. The average expression for each miRNA and the corresponding log₂ fold-
557 change were then graphed as a scatterplot. All tumor vs non-tumor miR-375 Graph: miR-375
558 expression was extracted for each primary colon tumor (n= 371) and solid normal colon tissue
559 (n=8). Expression of miR-375 was then graphed according to the tissue condition. Matched
560 tumor vs non-tumor miR375 Graph: Datasets representing the patient matched tumor and solid
561 normal tissue (n=8) were identified using the TCGA IDs. Expression for miR-375 was extracted
562 and graphed. Lines connecting data points link patient matched samples. Correlation analysis
563 graph: Significantly upregulated genes in primary colon tumor samples were identified as those
564 genes with an average expression above 1000 normalized counts in tumor (n=39) or non-tumor
565 (n=382), a Benjamini Hochberg adjusted P-value below 0.05, and a log₂ fold-change above 0.
566 Spearman correlation coefficients were calculated for each upregulated gene with miR-375 for
567 those samples with both RNA-seq and miRNA data (n=368). Each of the upregulated genes was

568 ranked based on its Spearman correlation coefficient with miR-375. The determined rank was
569 then graphed against the calculated correlation value.

570

571 ***Mouse Enteroid culture.*** Jejunal crypts were isolated from 3-5 month old male WT and 375-KO
572 B62J mice as previously described (Peck et al., 2017a). The isolated crypts (Day 0) were grown
573 into Reduced Growth Factor Matrigel (Corning, Corning, NY, cat. 356231). Advanced
574 DMEM/F12 (Gibco, Gaithersburg, MD, cat. 12634-028) supplemented with GlutaMAX (Gibco,
575 cat. 35050-061), Pen/Strep (Gibco, cat. 15140), HEPES (Gibco, cat. 15630-080), N2 supplement
576 (Gibco, cat. 17502-048), 50 ng/mL EGF (R&D Systems, Minneapolis, MN, cat.2028-EG), 100
577 ug/mL Noggin (PeproTech, Rocky Hill, NJ, cat. 250-38), 250 ng/uL murine R-spondin (R&D
578 Systems, cat. 3474-RS-050), and 10 mM Y27632 (Enzo Life Sciences, Farmingdale, NY, cat.
579 ALX270-333-M025) was added. For miRNA loss-of-function studies, miRCURY LNA Power
580 Inhibitor against mouse miR-375 (mmu-miR-375-3p) (Qiagen, Hilden, Germany, cat.
581 Y104101397-DFA) or Power Negative Control A (Qiagen, cat. YI00199006-DDA) was added at
582 500 nM on Day 0 and supplemented at 250 nM on Day 3. Enteroids at Day 5 were harvested for
583 RNA isolation or fixed in 4% (v/v) paraformaldehyde for whole mount staining. For studies
584 inhibiting Wnt and Yap1 function, enteroids were treated with IWP2 (Tocris Bioscience, Bristol,
585 UK, cat. 3533) and verteporfin (Sigma-Aldrich, St. Louis, MO, cat. SML0534-5ML),
586 respectively. Enteroid cultures were exposed to these inhibitors at Day 0 with the initial medium
587 and at Day 3 with the replenishment medium.

588

589 ***Whole mount enteroids immunostaining and imaging.*** The fixed mouse enteroids were
590 permeabilized with 0.5% (v/v) Triton X-100/PBS, washed with PBS containing 0.1% (w/v)

591 BSA/0.02% (v/v) TritonX-100/0.05% (v/v) Tween-20 and blocked with 10% (v/v) normal goat
592 serum. Primary antibodies were used to stain PH3 (rabbit anti- Phospho-Histone H3 (Ser10),
593 1:100, Cell Signaling, Danvers, MA, cat. 9701S). The staining was visualized by fluorescence
594 microscopy with fluorescent-conjugated secondary antibodies (goat anti rabbit Alexa Fluor 594,
595 1:400, ThermoFisher, Waltham, MA, cat. A-11034). Nuclei were counterstained with Hoechst
596 33348 dye (1:1000). The immunofluorescent staining was visualized and z-stack bright field
597 images were taken by a ZEISS Axiovert 200M inverted microscope (Zeiss, Jena, Germany).

598

599 ***CRISPR/Cas9 editing of mouse enteroids.*** The proximal half of the small intestine was
600 harvested from ~6-week-old C57BL/6 mice. Crypts were isolated and plated in Matrigel. Cells
601 were grown for 3-4 weeks to allow enteroids to form. Enteroids were then transfected with
602 CRISPR base editing tools and guide RNAs to make the APC^{Q883*} edit (Han et al., 2017). Cells
603 were then cultured in the absence of Rspo for ~2 weeks to select for Apc mutant cells.
604 Following selection, cells were frozen down.

605

606 ***Generation and infection of genetically modified lines of Drosophila.*** Esg-Gal4; UAS-GFP,
607 tub-Gal80^{TS} (Esg^{TS}, progenitor specific) (Micchelli and Perrimon, 2006) or Esg^{F/O} (Esg-Gal4,
608 UAS-GFP, tub-Gal80TS; UAS-FLP, act_{ftt}STOP_{ftt}-Gal4) (Jiang and Edgar, 2009) fruit flies were
609 crossed to UAS lines (BDSC 59916) for creating flies with miR-375 overexpression in Esg
610 stem/progenitor cells. Parental flies were crossed using ~15 female flies and 5 males. They
611 were then transferred during development in a 12:12 hour light/dark 18°C incubator. The
612 parental generation was removed after 5 days in the 18°C incubator to control for fly density of
613 the F1 progeny. Esg^{TS} or Esg^{F/O} flies were crossed to wild type line CantonS (BDSC: 64349) to

614 generate control flies. Parental lines were maintained at room temperature (~23°C) on standard
615 fly medium (50 g baker yeast, 30 g cornmeal, 20 g sucrose, 15 g agar, 5 mL 99% (v/v) propionic
616 acid mix, 0.5 mL 85% (v/v) phosphoric acid, 26.5 mL methyl paraben in 1L ethanol) in a 12:12
617 hours light/dark cycle. Oral infection of pathogen *Erwinia carotovora ssp. carotovora* 15
618 (*Ecc15*) was performed as previously described (Buchon et al., 2009). Orally treated flies were
619 incubated at 29°C until dissection for analyses.

620

621 ***Immunostaining of Drosophila midgut.*** The excised *Drosophila* midguts were fixed in 4%
622 (v/v) paraformaldehyde and washed with 0.1% (v/v) Triton X-100 in PBS. The samples were
623 then incubated for 1 hr in blocking solution (1% (w/v) bovine serum albumin, 1% (v/v) normal
624 donkey serum, and 0.1% (v/v) Triton X-100 in PBS) followed by overnight primary antibody
625 incubation and 2 hr secondary antibody staining. The primary antibody used in this study was
626 rabbit anti-PH3 (1:000, EMD Millipore, Burlington, MA, cat. 06-570). The secondary antibody
627 used in this study was donkey anti-rabbit-555 (1:2000, Thermo Fisher, Waltham, MA, cat. A-
628 31572). DAPI (1:50000) was used to visualize nuclei. Imaging was performed on a Zeiss LSM
629 700 fluorescent/ confocal inverted microscope (Zeiss, Jena, Germany).

630

631 ***Statistics.*** In most figure panels, quantitative data are reported as an average of biological
632 replicates ± standard error of the mean. In figure panels pertaining to whole mount
633 immunofluorescent staining in enteroids, quantitative data are reported as an average per
634 enteroid from an experiment ± standard error of the mean (enteroids from n=2-5 wells per
635 condition). In all analyses, statistical differences were assessed by two-tailed Student's t-test
636 with threshold P-value < 0.05, unless otherwise specifically noted.

637

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648

649 **Author Contributions**

650 Conceptualization, M.T.S., M.K., A.P.S., and P.S.; Methodology, M.T.S., M.K., A.P.S., and
651 P.S.; Formal Analysis, M.K., J.W.V., and W.A.P.; Investigation, M.T.S., M.K., A.P.S., J.W.V.,
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654 P.S.; Writing-Review & Editing, M.T.S., M.K., A.P.S., J.W.V., O.O.O., A.B., Y-H.H., C.M.D.,
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656 P.S.; Funding acquisition, J.W.V., Y-H.H., B.S., C.D., J.C.S., L.E.D., N.B., E.D.T., and P.S..

657

658 **Declaration of Interests**

659 The authors declare no competing interests.

660

661 **References**

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887

888 **Figure legends**

889

890 **Figure 1. miR-375 is highly enriched in Lgr5-High ISC's and stem-like LSP cells.** **A,**
891 Volcano plot of differentially expressed microRNAs assessed by small RNA-seq analysis of
892 isolated jejunal crypts relative to isolated jejunal villi from 3-5 months old C57BL/6 male mice
893 (n=4). Only microRNAs with an RPMMM > 1000 in either crypt or villi were included in the
894 analysis. Significant ($P < 0.05$; Student's t-test) microRNAs with fold changes above 2.5 in
895 intestinal crypts are colored red, while significant microRNAs with fold changes below -2.5 in
896 intestinal crypts are colored blue. miR-375 is colored green. **B,** Venn diagram of the top 20
897 expressed microRNAs in Sox9-Low (blue), Lgr5-High (green), and Cd24-Low (red) FACS
898 sorted cells. **C,** RT-qPCR data of miR-375 expression in jejunal crypts (n=2) of C57BL/6 mice
899 relative to jejunal villi (n=2). **D,** Enrichment of the 14 microRNAs common to stem cell
900 fractions in Lgr5-High cells versus Sox9-Neg cells. miR-375-3p is identified in black. **E,**

901 Enrichment of the 14 microRNAs common to stem cell fractions in Sox9-Low cells versus Sox9-
902 Neg cells. miR-375-3p is identified in black. **F**, RT-qPCR data of miR-375 expression of LSP
903 sorted cells (n=3) relative to USP sorted cells (n=3). **G**, RT-qPCR data of miR-375 expression
904 in jejunal crypts of 11-17 months old C57BL/6 male mice (Old, n=6) relative to 2-5 months old
905 mice (Young, n=6). **H**, RT-qPCR data of miR-375 expression in jejunal enteroids established
906 from tissue of WT B62J mice treated with 1 μ M of Wnt antagonist IWP2 (n=3) relative to the
907 mock condition (n=3). Representative of three independent experiments. **I**, Volcano plot of
908 differentially expressed microRNAs assessed by small RNA-seq analysis of mouse jejunal
909 enteroids with a Wnt activating APC mutation (APCmut) relative to non-mutated C57BL/6
910 wildtype (WT). Only microRNAs with a normalized count > 1000 in either WT or APCmut
911 were included in the analysis. Significant (adjusted $P < 0.01$; DESeq2) microRNAs with fold
912 change above 3 in APCmut enteroids are colored red, while significant microRNAs with fold
913 change below -3 in APCmut enteroids are colored blue. miR-375 is identified in blue. **J**, RT-
914 qPCR data of miR-375 expression in mouse jejunal enteroids with a Wnt activating APC
915 mutation (APCmut, n=2) or a Wnt activating β -catenin mutation (β -Cat-mut, n=2) relative to
916 non-mutated C57BL6/J (WT, n=2). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by two-tailed
917 Student's t-test. RQV, relative quantitative value. RPM, reads per million miRNAs mapped.

918

919 **Figure 2. miR-375 deficiency increases small intestinal tuft cell numbers and enhances**
920 **resistance to helminth *Heligmosomoides polygyrus* infection.** **A**, UMAP scatterplot of 12
921 months old jejunal crypt cells from WT (n=2) and 375-KO (n=2) B62J mice analyzed by single
922 cell RNA-seq. Cell clusters pertaining to the seven primary intestinal cell types are colored and
923 labeled. **B**, Hierarchical clustering based on gene expression of classic markers of the seven

924 small intestinal cell types. Red indicates enriched expression relative to all cells whereas as blue
925 indicates de-enrichment of expression. **C**, Bar plot of the ratio of the percentage of 375-KO-
926 derived cells within the various intestinal cell type clusters to the percentage of those derived
927 from WT mice. **D**, Bar plot of transcript levels of tuft cell marker genes (*Pou2f3*, *Gfi1b*, *Ascl2*,
928 *Dclk1*, *Trpm5*, and *Rgs13*) across all 375-KO jejunal crypt cells relative to WT. **E**, Bar plot of
929 the percentage of eosinophils within isolated immune cell suspensions from FACS analyzed
930 small intestinal tissue from WT (n=10) and 375-KO (n=10) mice after 14 days of infection with
931 the helminth *Heligmosomoides polygyrus*. **F**, Bar plot of the number of *Heligmosomoides*
932 *polygyrus* worms collected from the small intestinal lumen of WT (n=7) and 375-KO (n=7) mice
933 after 14 days of infection (two representative experiments out of a total of three are shown; red –
934 experiment #1, blue – experiment #2). * P < 0.05, ** P < 0.01, *** P < 0.001 by two-tailed
935 Student's t-test.

936

937 **Figure 3. miR-375 deficiency augments the Wnt signaling pathway in ISCs and enhances**
938 **the irradiation-induced regeneration response.** **A**, Ranked bar plot of the -log₁₀ P-values of
939 the ten most enriched transcription factor regulators of the genes upregulated in the ISC cluster
940 of 375-KO mice relative to WT mice. Established Wnt pathway transcription factors are denoted
941 in orange. TRRUST is a manually-curated database of mouse and human transcriptional
942 regulatory networks. **B**, Bar plots of the expression levels of Wnt signaling genes *Ccnd2*, *Cd44*,
943 *Ascl2*, *Myc*, *Cldn2*, and *Trim65* within each of the cell clusters identified by scRNA-seq analysis
944 in WT and 375-KO. **C**, UMAP scatter plots of a subset of genes shown in (B). Level of
945 expression for the Wnt signaling genes *Ascl2*, *Myc*, and *Trim65* is shown in purple, while non-
946 expressing cells are shown in grey. Only the ISC cluster is shown and the percentage of cells in

947 the ISC cluster that are positive for the assayed gene is indicated in the top right corner in red.
948 **D**, Bar plot of the mid-jejunal crypt depth for WT (n=4-5 mice) and 375-KO mice (n=4-7 mice)
949 1 day and 2.5 days after irradiation. **E**, Photomicrographs (x200) of H&E stained mid-jejunum
950 in WT and 375-KO mice 1 day and 2.5 days post-irradiation (p.i.). Two different individual 375-
951 KO mice are shown for 1 day and 2.5 days post-irradiation (p.i.). Yellow scalebar represents 50
952 μm . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-tailed Student's t-test.

953

954 **Figure 4. *In vivo* genetic loss and *ex vivo* knockdown of miR-375 promotes intestinal**
955 **epithelial survival and proliferation.** **A**, Plot of number of buds per enteroid of jejunal crypts
956 derived from 375-KO relative to WT. **B**, Phase-contrast microphotographs (x400) of jejunal
957 enteroids established from crypts of WT and 375-KO mice. Enteroid buds are indicated by red
958 arrowheads. Yellow scalebar represents 50 μm . **C**, Plot of relative number of surviving jejunal
959 enteroids from WT and 375-KO mice under conditions of mock treatment (n=3) or treatment
960 with 1 μM of IWP2 (n=3). Values are expressed relative to mock treated WT mouse jejunal
961 enteroids. Representative of three independent experiments (n=3-5 wells/group). **D**, Phase-
962 contrast microphotographs (x50) of jejunal enteroids established from crypts of WT and 375-KO
963 mice that were either mock treated or treated with 1 μM of IWP2. Yellow scalebar represents
964 500 μm . **E**, Plot of number of buds per enteroid of wildtype (WT) B62J mouse jejunal enteroids
965 that were either under mock, locked nucleic acid (LNA)-scrambled (LNA-scr) or anti-miR-375
966 LNA (LNA-375) treated conditions (n=36 Mock, n=36 LNA-scr, n=31 LNA-375 enteroids
967 examined). **F**, Phase-contrast microphotographs (x400) of WT mouse jejunal enteroids that were
968 either mock, LNA-scr, or LNA-375 treated. Buds are indicated by red arrowheads. Yellow
969 scalebar represents 50 μm . **G**, Plot of number of PH3+ cells per enteroid of mock, LNA-scr, and

970 LNA-375 treated WT mouse jejunal enteroids (n=126 Mock, n=72 LNA-scr, n=58 LNA-375
971 enteroids examined). Yellow scalebar represents 50 μ m. **H**, Fluorescent microphotographs
972 (x400) of representative enteroids from WT mock, LNA-scr, and LNA-375 treated enteroid
973 cultures. Hoechst-stained nuclei are shown in blue. PH3+ cells are shown in red and indicated
974 by yellow arrowheads. Yellow scalebar represents 50 μ m. **P < 0.01, *** P < 0.001 by two-
975 tailed Student's t-test.

976

977 **Figure 5. *In vivo* overexpression of miR-375 in *Drosophila* midgut ISCs reduces**
978 **proliferation.** **A**, Sequence alignment of mature miR-375 for *Homo sapiens* (hsa-miR-375-3p),
979 *Mus musculus* (mmu-miR-375-3p), *Sus scrofa* (ssc-miR-375), and *Drosophila melanogaster*
980 (dme-miR-375-3p). The conserved seed sequences are depicted in red. **B**, Confocal
981 microphotographs (x200) of representative midgut sections of *esg^{F/O}>CantonS* and
982 *esg^{F/O}>375OE Drosophila* in baseline, unchallenged (UC) conditions. Nuclei are shown in blue
983 (DAPI). *Esg*+ progenitor cells (GFP+) and their differentiated progeny (GFP+) are shown in
984 green. **C**, Confocal microphotographs (x200) of representative midgut sections of
985 *esg^{TS}>CantonS* and *esg^{TS}>375OE Drosophila* in baseline, UC conditions. Nuclei are shown in
986 blue (DAPI). *Esg*+ progenitor cells (GFP+) are shown in green. **D**, Confocal microphotographs
987 (x200) of representative midgut sections of *esg^{TS}>CantonS* and *esg^{TS}>375OE Drosophila*
988 infected with *Erwinia carotovora carotovora* 15 (*Ecc15*) for 12 h. Nuclei are shown in blue
989 (DAPI). *Esg*+ progenitor cells (GFP+) are shown in green. Yellow scalebar indicates 50 μ m. **E**,
990 Bar plots of total PH3+ cells in the entire midgut of control *D. melanogaster* (*esg^{TS}>CantonS*,
991 n=22-23) and *D. melanogaster* overexpressing miR-375 only in *esg*+ progenitor cells

992 (*esg*^{TS}>375OE, n=23-26) under UC conditions or orally infected with *Ecc15* for 12 h. *** P <
993 0.001 by two-tailed Student's t-test.

994

995 **Figure 6. miR-375 influences enteroid growth in part by regulating Yap1 signaling. A,**

996 Scatter plot of small RNA-seq-determined expression of miR-375 and RNA-seq-determined

997 expression of *Yap1* in jejunal Sox9-Low cells derived from matched mice that were in a diet

998 study in which they were fed either chow (Chow, red) (n=4) or a high fat diet (HFD, green)

999 (n=4) for a prolonged period (16-20 weeks), or were from a study in which the mice were either

1000 conventionalized (CV, blue) (n=4) or germfree (GF, purple) (n=4) (Peck, Mah et al.). Pearson

1001 correlation analysis revealed a strong inverse correlation (R= -0.808). **B,** Plot of number of buds

1002 per enteroid for WT and 375-KO mouse jejunal enteroids that were either mock treated or treated

1003 with 2 μ M or 5 μ M of verteporfin. **C,** Phase-contrast microphotographs (x400) of WT and 375-

1004 KO mouse jejunal enteroids treated with either 2 μ M or 5 μ M of verteporfin. Yellow scalebar

1005 indicates 25 μ m. **D,** Plot of number of surviving enteroids of WT (n=3) and 375-KO (n=3)

1006 mouse jejunal enteroids treated with either 2 μ M or 5 μ M of verteporfin. **E,** Phase-contrast

1007 microphotographs (x50) of WT and 375-KO mouse jejunal enteroids treated with either 2 μ M or

1008 5 μ M of verteporfin. Yellow scalebar indicates 500 μ m. *P < 0.05, **P < 0.01, *** P < 0.001 by

1009 two-tailed Student's t-test. RPMMM, reads per million mapped to microRNAs. RQV, relative

1010 quantitative value.

1011

1012 **Figure 7. Intestinal cancer is associated with depressed expression of miR-375.**

1013 **A,** Scatter plot using data from TCGA database to display the fold change of miRNA expression

1014 in human primary colon adenocarcinoma (n=371) to human colonic non-tumor tissue (n=8).

1015 miR-375 is indicated in red. **B**, Bar plot of miR-375 expression in colon non-tumor tissue (n=8)
1016 and primary colon adenocarcinoma (n=371) from TCGA data. **C**, Dot plot of miR-375
1017 expression from TCGA colon non-tumor tissue (n=8) and matched primary, colon
1018 adenocarcinoma (n=8) with lines connecting tumors derived from the same patient. **D**,
1019 Upregulated genes in TCGA primary colon adenocarcinoma that are predicted miR-375 targets
1020 in mouse and human were ranked according to the calculated spearman correlation coefficient to
1021 identify inversely correlated genes with miR-375 expression. *YAPI* is indicated in red. **E**,
1022 Working model of miR-375 expression and function in the intestinal epithelium. *P < 0.05, ***
1023 P < 0.001 by two-tailed Student's t-test. RQV, relative quantitative value. RPMMM, reads per
1024 million mapped to microRNAs.
1025

Figure 1

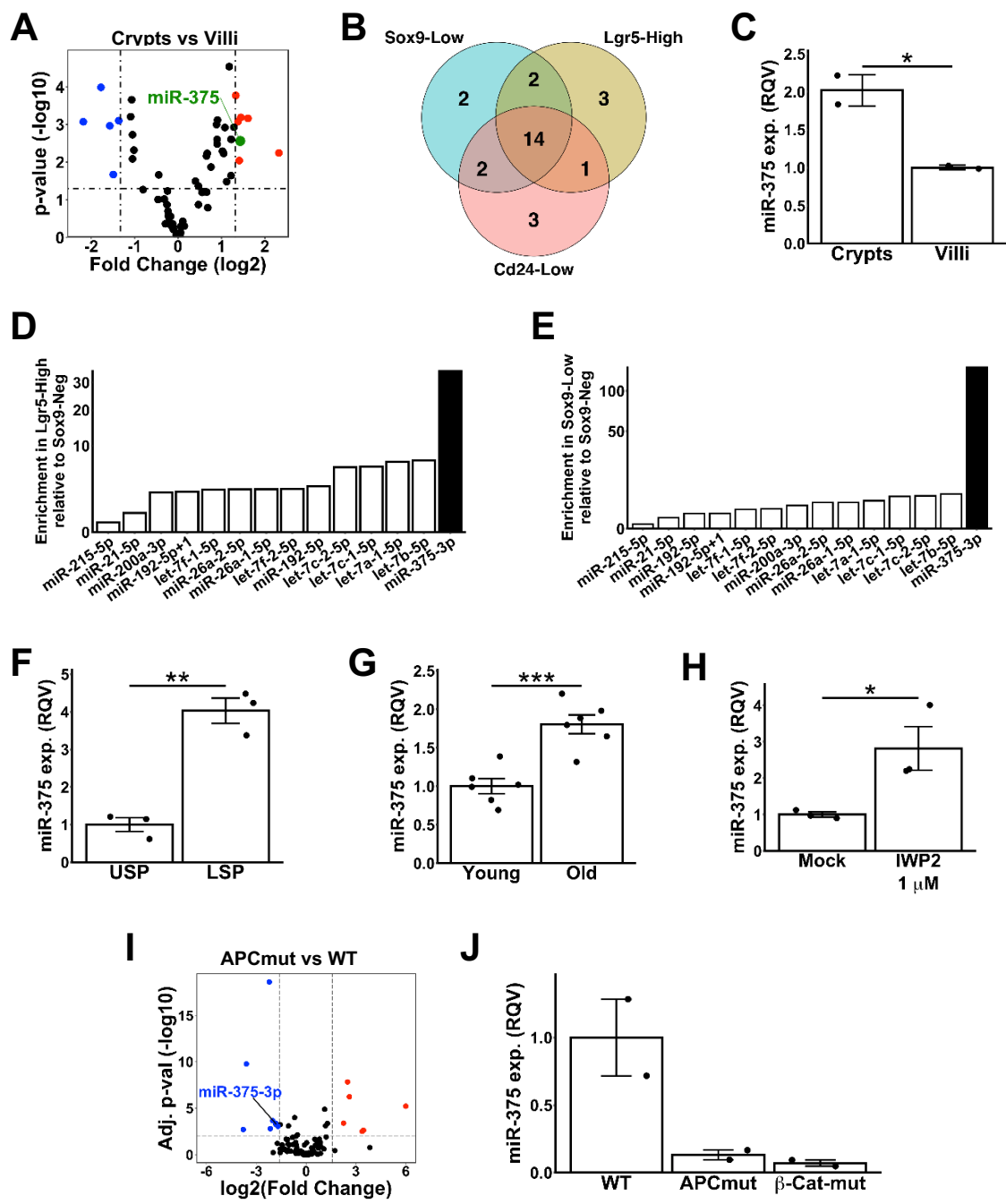


Figure 2

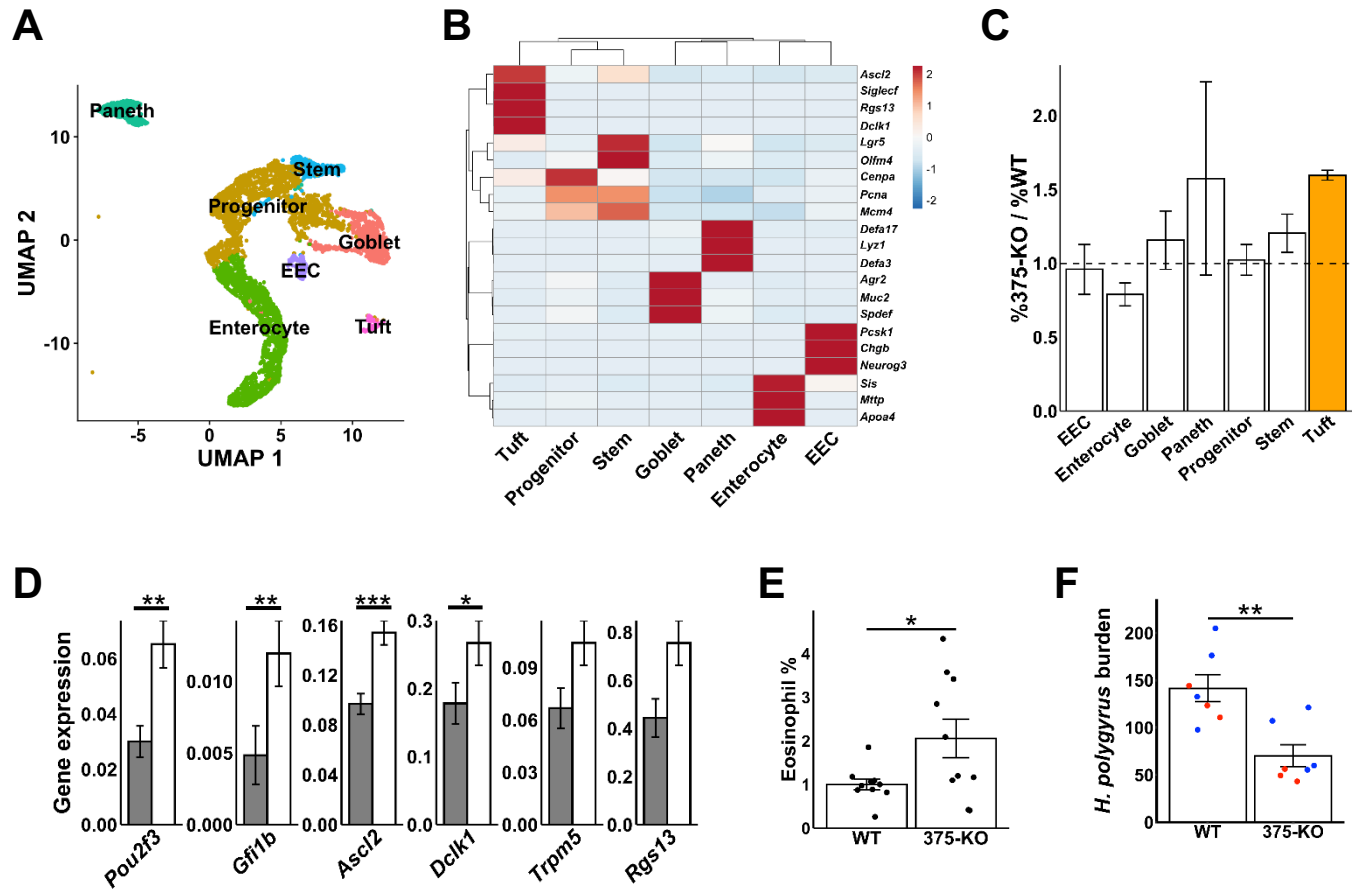


Figure 3

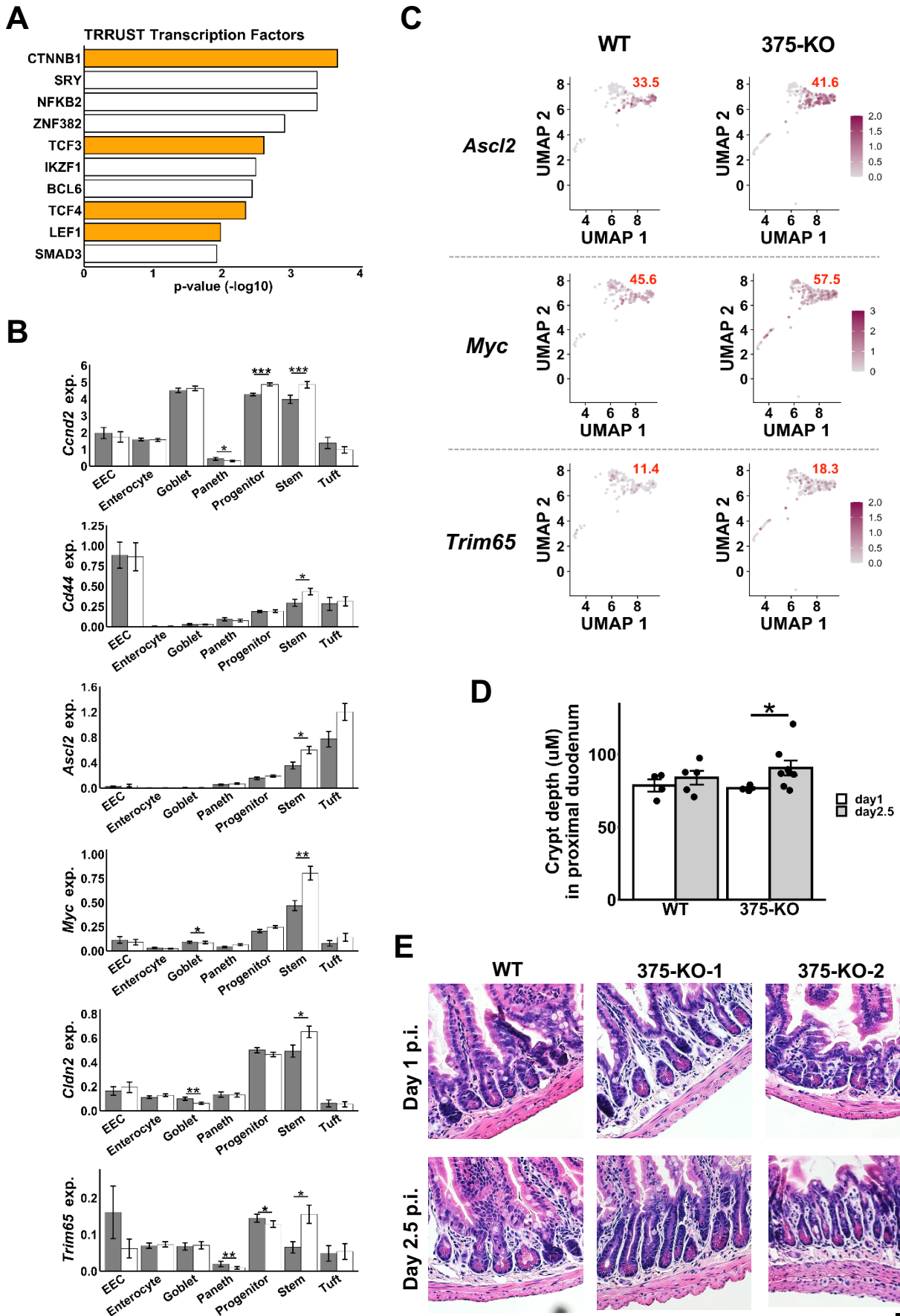
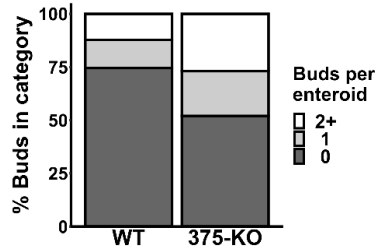
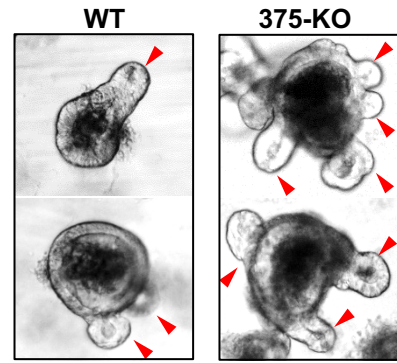


Figure 4

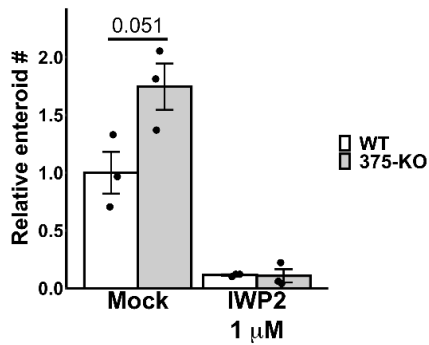
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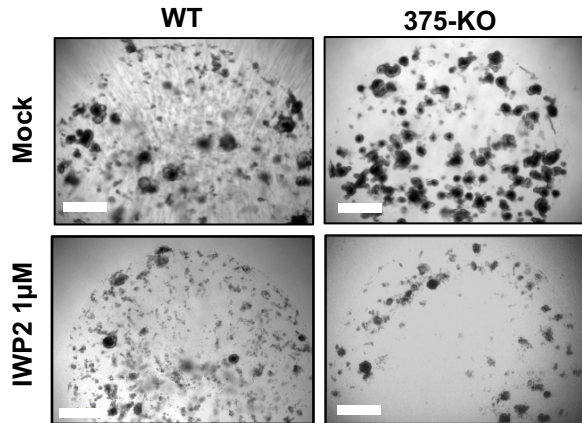
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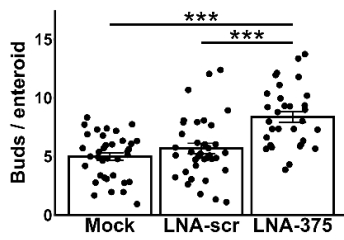
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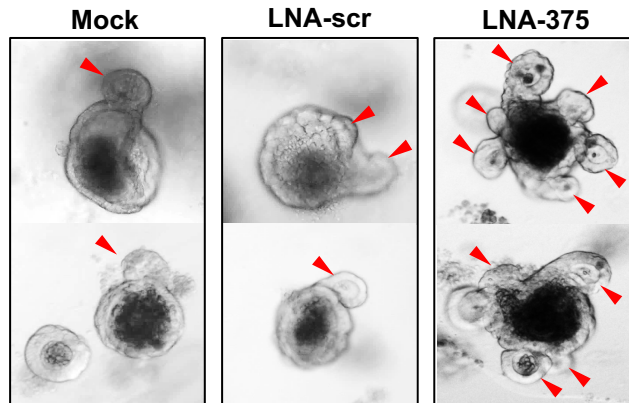
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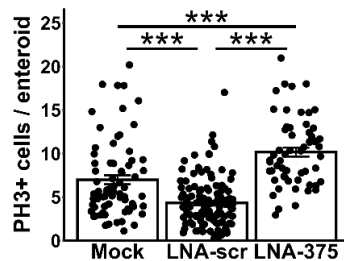
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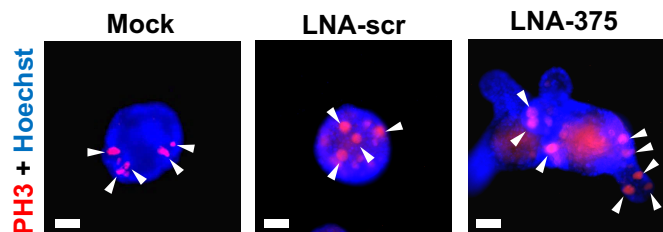
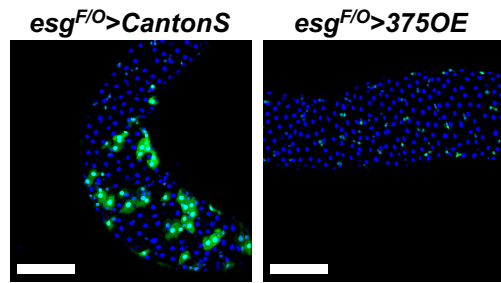


Figure 5

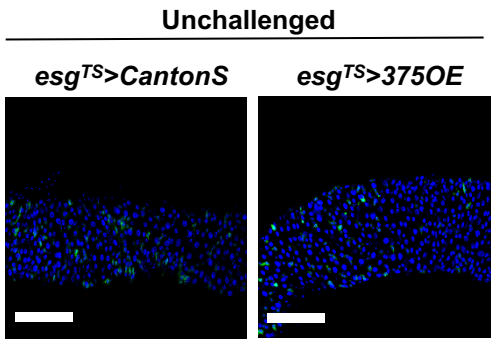
A

hsa-miR-375-3p 5'-UUUGUUCGUUCGGCUCGCGUGA-3'
mmu-miR-375-3p 5'-UUUGUUCGUUCGGCUCGCGUGA-3'
ssc-miR-375 5'-UUUGUUCGUUCGGCUCGCGUGA-3'
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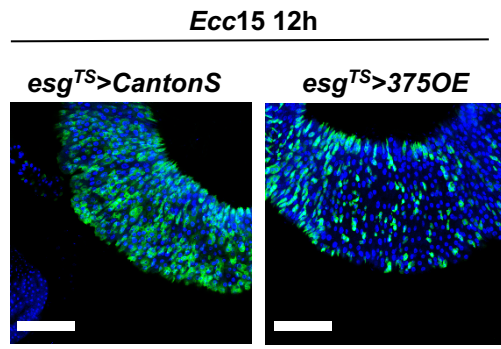
B



C



D



E

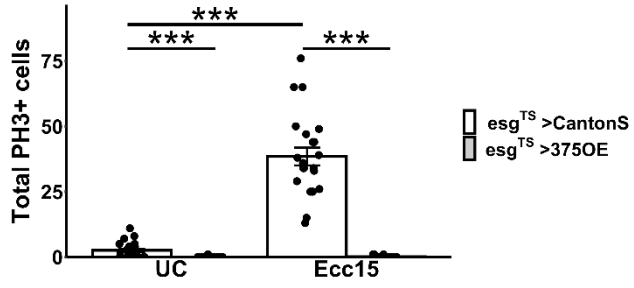


Figure 6

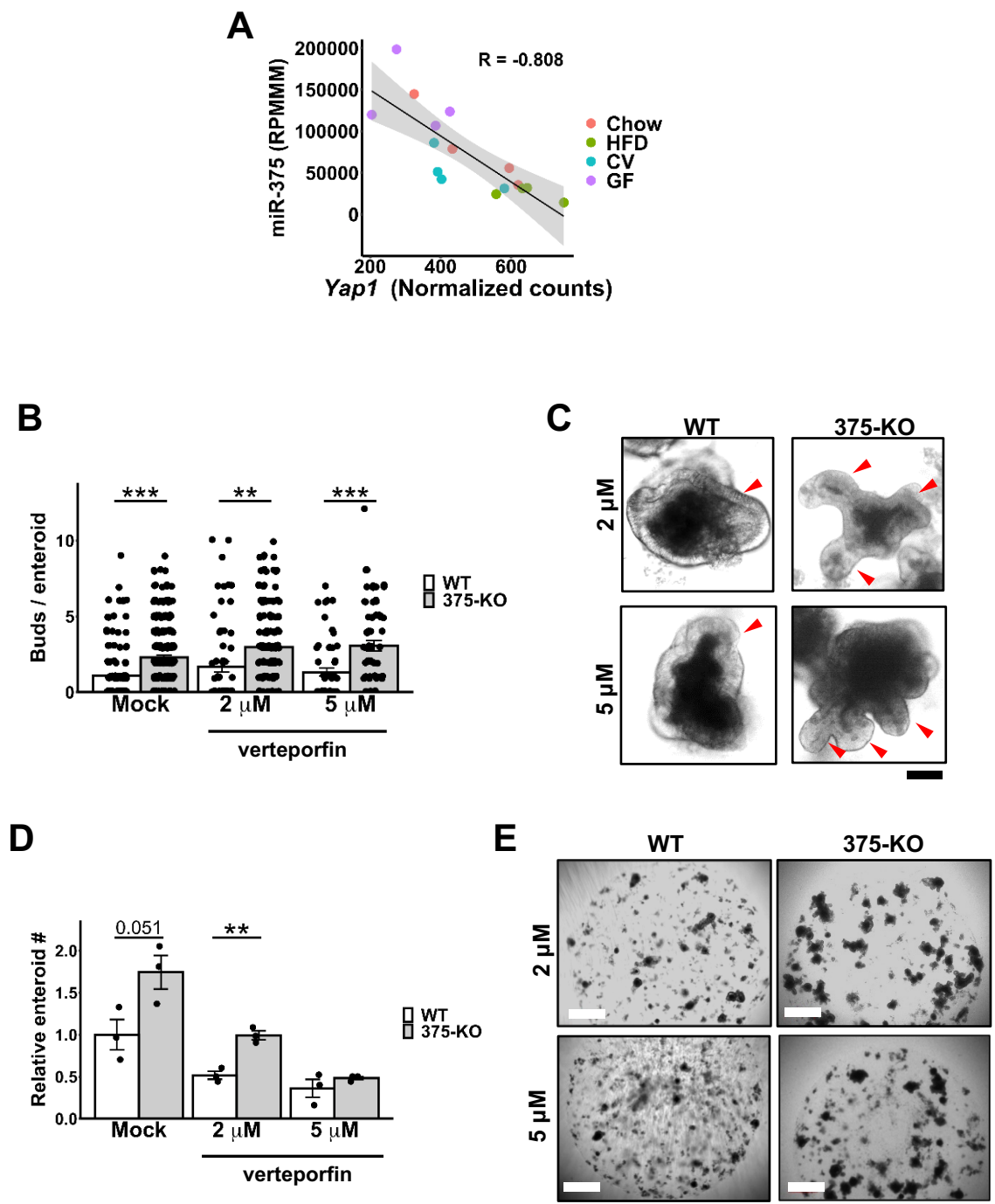


Figure 7

