1 **Dynamic intestinal stem cell plasticity and lineage remodeling by a nutritional**
2 **environment relevant to human risk for tumorigenesis**

3

4 **Short title: Stem Cells and Lineages in Tumor Risk**

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39 repositories; reagents will be made available to other investigators upon request to the
40 corresponding author.

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42 **Synopsis:** Mouse nutrient exposures reflecting those in humans dynamically remodel mouse
43 stem cells and lineages, uniquely causing sporadic intestinal and colon tumors as in humans.
44 Pathogenic mucosal alterations parallel aspects of human inflammatory bowel disease and
45 plasticity of stem cell populations in human tumors.

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47 **Key words**: Diet; tumor risk; stem cells; homeostasis; epigenetic reprogramming
48

Abstract

NWD1, a purified rodent diet establishing mouse exposure to key nutrients recapitulating

levels that increase risk for intestinal cancer, reproducibly causes *sporadic* intestinal and colonic

tumors in the mouse, reflecting human etiology, incidence, frequency and lag with

developmental age. Complex NWD1 reprogramming of stem cells and lineages was

deconvolved by bulk and scRNAseq, scATACseq, functional genomics and imaging. NWD1

extensively, rapidly, and reversibly reprogrammed $Lgr5^{hi}$ stem cells, epigenetically down-

regulating *Ppargc1a* expression, altering mitochondrial structure and function. This suppressed

$Lgr5^{hi}$ stem cell functions and developmental maturation of $Lgr5^{hi}$ cell progeny as cells

progressed through progenitor cell compartments, recapitulated by *Ppargc1a* genetic

inactivation in $Lgr5^{hi}$ cells *in vivo*. Mobilized *Bmi1+, Ascl2^{hi}* cells adapted lineages to the

nutritional environment and elevated antigen processing and presentation pathways, especially

in mature enterocytes, causing chronic, pro-tumorigenic low-level inflammation. There were

multiple parallels between NWD1 remodeling of stem cells and lineages with pathogenic

mechanisms in human inflammatory bowel disease, also pro-tumorigenic. Moreover, the shift to

alternate stem cells reflects that the balance between Lg5 positive and negative stem cells in

supporting human colon tumors is determined by environmental influences. Stem cell and

lineage plasticity in response to nutrients supports historic concepts of homeostasis as a

continual adaptation to environment, with the human mucosa likely in constant flux in response

to changing nutrient exposures. Thus, although oncogenic mutations provide a competitive

advantage to intestinal epithelial cells in clonal expansion, the competition is on a playing field

dynamically sculpted by the nutritional environment, influencing which cells dominate in mucosal

maintenance and tumorigenesis.

Introduction:

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77   Plasticity of intestinal cell function underlies seemingly rigid mucosal structural and functional

78   organization. For example, following damage or targeted ablation of Lgr5^{hi} intestinal stem cells

79   (ISCs), multiple cell types can dedifferentiate to function as stem-like cells, interpreted as a

80   damage response to restore homeostasis (1,2). However, original concepts of homeostasis, by

81   Claude Bernard in the 1860's as the "milieu interieur", and Walter Thompson in the 1940's in

82   coining the term, defined homeostasis as dynamic continual tissue adaptation to its environment,

83   not a single target state returned to following disruption (3). Indeed, we reported that plasticity of

84   intestinal cells to function as stem cells is determined by dietary patterns (4-6) and a recent report

85   documents rapid mucosal adaptation to a high fat chow diet by upregulating fat metabolism

86   pathways (7). Thus, response to nutrients is complex, involving not only adaptive lineage

87   reprogramming, but a major impact on which cells function as stem cells.

88   The dietary induced stem cell plasticity was in response to NWD1, a purified diet that uniquely

89   causes sporadic intestinal and colon tumors, defined broadly as tumors without clear genetic

90   inheritance and without exposure to known carcinogens. These comprise the majority of human

91   intestinal tumors and are strongly linked to human dietary exposures (4,6,8,9). Moreover, in this

92   model, stem cell reprogramming by NWD1 is a function of nutrient interactions (6), of major

93   importance since scarcity of dietary studies compared to studies of individual nutrients has been

94   emphasized, and it is diet overall that influences cell physiology, disease risk, and progression

95   (10-12). This is not surprising since a vast literature establishes that even strongly penetrant onco-

96   and tumor suppressor genes are always context dependent, with very different effects in different

97   tissues.

98   There are many strategies for modeling the complex and variable human diet. In the NWD1

99   model, the principle of nutrient density to adjusts multiple key nutrients to recapitulate exposure

100   levels of each linked to elevated risk for colon cancer in broad segments of high-risk western

101   populations (13,14). Fat is at 25%, reflecting levels characterizing human high risk, rather than

102   60% that causes metabolic alterations differing from those caused by level better reflecting human

103   exposure (15,16). NWD1 also is lower in calcium, vitamin D, methyl donors and fiber, key risk

104   factors for human colon cancer (17). This combination of nutrient risk factors accelerates and

105   amplifies tumor phenotype in multiple genetically initiated models (18-22), and is unique in

106   reproducibly causing *sporadic* tumors of the small and large intestine of wild-type mice, reflecting

107   the etiology, incidence, frequency and lag with developmental age of human colon cancer, the

108   majority of the disease (14,22-24) and that linked to dietary exposures (25). NWD1 fed mice are

109   not obese, but exhibit reduced energy expenditure without altered food consumption or physical

110   activity, causing glucose intolerance, and increased pro-inflammatory cytokines (26). NWD1

111   elevates Wnt signaling throughout the small and large intestine (27-29), and expands the

112   proliferative compartment (30,31), as in humans at elevated tumor risk (32-35). It is striking that

113   in NWD1 fed mice, Lgr5^{hi} ISCs are repressed in supporting mucosal maintenance, show reduced

114   mutation accumulation, and reduced efficiency in producing tumors on introduction of an initiating

115   *Apc* mutation, with these functions increasing for Bmi1+ cells (4,6,8,9). These data anticipate the

116   recent report that both Lg5 positive and negative stem cells support human colon tumor growth,

117   the balance determined by environmental factors (36), emphasizing significance of NWD1 shifting

118   which cells function as stem cells in the mucosa.

119   Here single cell RNAseq, single cell ATACseq, functional genomics and imaging, with rigorous

120   and novel bioinformatic and statistical approaches, de-convolve complex cellular and molecular

121   remodeling of the mucosa increasing risk for the sporadic tumors caused by NWD1. This

122   establishes major new insight into dietary induced risk for sporadic tumors: epigenetic remodeling

123   of chromatin structure of key genes, including a fundamental role of *Ppargc1a* down regulation in

124   altering mitochondrial structure and function specifically in Lgr5^{hi} ISCs; rapidity and reversibility of

125   reprogramming by dietary shift; identity of the cell population mobilized by diet to maintain the

126   mucosa; and elevation of pathways of antigen processing and presentation linked to pro-

127   tumorigenic inflammation. Remarkably, multiple cell and molecular alterations in NWD1 fed wild-

128   type mice parallel pathogenic mechanisms in human inflammatory bowel disease (IBD) (37-39).

129   Therefore, while oncogenic mutations provide a stem cell with a competitive advantage for clonal

130   expansion (1), this competition is carried out on a playing field sculpted and continually remodeled

131   by the nutritional environment, with a major effect on outcome.

132

133   **Results:**

134   **NWD1 Impact on Lgr5hi intestinal stem cells (ISCs):**

135   Bulk RNAseq analyzed Lgr5hi cells from *Lgr5EGFP-creER* mice fed NWD1 or AIN76A control diet

136   for 3 or 12 months from weaning (**Fig 1A**: Arms 1, 2), or 3 months NWD1 then switched to

137   AIN76A and sacrificed at 12 months (Arm 3 – crossover). Of approximately 8,000 genes

138   expressed in isolated Lgr5hi cells, 164 and 63 increased or decreased, respectively, by feeding

139   NWD1 for 3 months (change ≥50% with *P≤0.01* (**Fig S1A**). This increased >3-fold at 12 months

140   to 551 and 229, but reverted to the more limited perturbation in mice fed NWD1 for 3 months

141   switched back to control AIN76A until 12 months (**Fig S1A**). At the pathway level (GSEA),

142   NWD1 significantly up- and down- regulated 25 and 30 pathways, respectively, in Lgr5hi cells at

143   3 months, many alterations persisting to 12 months of feeding, and reversed upon shift back to

144   control AIN76A diet (**Fig S1B**). Thus, NWD1 had a major effect on transcriptional profile of

145   Lgr5hi cells.

146   The Oxphos and the TCA cycle pathways were down-regulated by NWD1 at 3 months,

147   persisted until 12 months, and reversed by 12 months after switching back to control diet at 3

148   months (**Fig S1B, S2**), necessary for intestinal *Lgr5hi* cells, hematopoietic, and embryonic cells

149   to function as stem cells (40-44). NWD1 down-regulated 58 of 132 genes in the Oxphos

150   pathway at 3 months, confirmed in an independent cohort of mice also fed NWD1 or AIN76A for

151   3 months (6), with mean Oxphos gene expression decreased by 14% and 11% in the two data

152   sets (**Fig S3A**). Down regulation of most Oxphos genes was modest, but the altered profile was

153   highly significant ($P=10^{-26}$, Fisher's exact test in comparison to the 8,000 member expressed

5

154   gene set). The down regulated profile and pathway persisted at 12 months (**Fig S1B, S2, S3B**),

155   encompassing multiple genes encoding subunits of the mitochondrial electron transport chain

156   (**Fig S3C).** Greatest down regulation in both cohorts were *Cox6a2* and *Cox7a1* of cytochrome

157   oxidase, the terminal respiratory chain enzyme, and *Sdhb*, and *Sdhd* of complex II, succinic

158   dehydrogenase (**Fig S3A**), down-regulated in CRC and other cancers causing succinate

159   accumulation and tumor development (45,46). In crossover to control diet (Arm 3), 52 (82%) of

160   down-regulated Oxphos genes increased towards control levels, also highly significant (**Fig**

161   **S3B;** *P=10^{-18}*), reflecting GSEA reversal (**Fig S1B, S2)**.

162   *Ppargc1a*, encoding Pgc1a, is a master regulator of metabolism and mitochondrial

163   biogenesis (47,48) and *Ppargc1a* overexpression elevates mitochondrial biogenesis and

164   function of colonic epithelial cells, altering their fate (49). Importantly, feeding NWD1 for 3

165   months reduced *Ppargc1a* expression in Lgr5^{hi} cells by ≥60% in both mouse cohorts (**Fig 1B,D**

166   *P=0.008 and 0.03*), similarly decreased at 12 months of NWD1 feeding *(P=0.06*), and

167   switchover back to control AIN76A produced levels indistinguishable from mice continuously fed

168   AIN76A from weaning (**Fig 1C**). By immunohistochemistry, the encoded protein, Pgc1a, was

169   highest in crypt cells of control fed mice, but much lower in crypts of NWD1 fed mice, paralleling

170   decreased *Ppargc1a* expression in Lgr5^{hi} stem cells (**Fig 1E**). Quantification across multiple

171   crypts-villi established decreased Pgc1a in crypts of NWD1 fed mice (*P<0.01*), but much lower

172   expression, and no difference with diet, at any position above 20 microns of the crypt base (**Fig**

173   **1F**). Further confirmation was decreased *Ppargc1a* expression in Lgr5^{hi} cells, but not in Bmi1+

174   cells most abundant in cells further up into the villi (**Fig 1G**). Quantitatively, cristae density in

175   crypt base epithelial cells decreased in NWD1 fed mice (*P<01*), with no change in cristae

176   density in Paneth cells at the base, or in cells higher up (**Fig 1H,I**). Finally, GFP^{hi} cells of

177   *Lgr5^{EGFP}* mice fed NWD1 decreased by 60% (**Fig 1J**, *P=0.03*) and their retention of mitotracker

178   dye decreased (**Fig 1K**, *P<0.0001*), an index of mitochondrial function. Therefore, NWD1, in

179   lowering *Ppargc1a* expression, its encoded Pgc1a protein, and expression of many genes

180   encoding multiple proteins of the electron transport chain, altered mitochondrial structure and

181   membrane potential in stem cells at the crypt base.

182   **Mechanistic role of *Ppargc1a* down regulation**: *Ppargc1a* was inactivated hetero- or

183   homozygously in Lgr5^{hi} cells of mice fed control diet (*Lgr5$^{EGFP-cre:ER-}$, Ppargc1a$^{loxp/loxp\ or\ loxp/+}$,

184   Rosa26^{tom}* mice). 3 days after homozygous inactivation, effects of feeding NWD1 were

185   phenocopied: Lgr5+ cell lineage tracing was significantly suppressed (**Fig 2A,B,** *P=0.02*), and

186   CBC cell cristae density significantly decreased (*P<0.05*) with no alteration in Paneth or in villus

187   cells (**Fig 1H,I**).

188   To define how this remodels stem cells and the mucosa, scRNAseq analyzed FACs isolated

189   intestinal epithelial cells (Epcam+, CD45neg) from mice 3 days after *Ppargc1a* inactivation in

190   Lgr5^{hi} cells. 20 clusters were identified and aligned with intestinal cell types and lineages (**Fig

191   2C -** (Methods)). A minor cluster could not be aligned (UK; <1% of cells in any single cell

192   library). Cell proportion in each cluster was similar among genotypes, but with increased early

193   enterocyte populations (EC1 and EC2) by heterozygous inactivation that increased further and

194   was statistically significant with homozygous inactivation (**Fig 2D**). Trajectory analysis placed

195   EC1 proximal to Dividing cells, EC2 emerging on that trajectory, and other EC cells and

196   lineages arising further down-stream (**Fig 2E-G**). This predicted trajectory using Monocle was

197   validated by interrogating markers that identify physical cell position along the crypt-villus axis

198   (**Fig S4A,** (50)). Therefore, decreased EC1 and 2 cell number within 3 days of *Ppargc1a*

199   inactivation in Lgr5^{hi} ISCs was consistent with these cells being the most immediate cells

200   emerging from the progenitor cell compartments.

201   Repressed mitochondrial function suppresses ability of Lgr5^{hi} cells to function as stem cells

202   (40-44), mitochondrial function determines balance between stem cells retaining self-renewal

203   capacity or differentiating (51), and mitochondrial fusion and hence activation is necessary for

204   intestinal stem cell progeny to differentiate (52). Therefore, impact of *Ppargc1a* inactivation in

205   Lgr5^{hi} cells on cell progression through progenitor cell compartments was determined (**Fig2 E-**

206   **G**). As expected, progressive maturation of progenitor cells began at the Stem cluster, with

207   maturation of progenitor cells through branch points giving rise to committed lineages. At branch

208   point "a", developmental trajectory from the Replicating 1 cell compartment (R1) bifurcates to

209   secretory goblet cell lineages or R2 cells (red arrow, **Fig2 E-G**). For both WT and *Ppargc1a* het

210   mice, R1 cells were the major cell type at branch point "a" with few or no Stem cells (**Fig 2H**).

211   Thus, at this early branch point, developmental transition from Stem to R1 had taken place.

212   However, with homozygous *Ppargc1a* inactivation, 100% of cells at "a" were still identified as

213   stem cells, a major, highly significant, repression of re-programming with maturation from Stem

214   to R1 cells (**Fig 2H**; *P=10^{-5}*, by a weighted linear regression based on cell number at each

215   cluster for each mouse). The altered cell distribution at branch "a" with homozygous inactivation

216   was characterized by significant suppression of Oxphos, TCA cycle, cell cycle, and glycolysis

217   pathways (**Fig 2I**), reflecting the altered mitochondrial structure and function with *Ppargc1a*

218   homozygous inactivation1 (**Fig 2H,I**).

219   With further progression along the validated trajectory, branch point "b" was transition from

220   R2 to enteroendocrine and Tuft cells (**Fig 2E-G**). At "b", most cells in WT or het mice had

221   progressed to the R2 compartment, but with homozygous *Ppargc1a* inactivation, 90% remained

222   in R1 (*P=0.016;* **Fig 2J**), with highly significant repression of the Oxphos pathway persisting

223   (**Fig 2K**). Therefore, homozygous *Ppargc1a* inactivation altered Stem cell maturation to R1

224   cells, and repressed transition out of R1 with down-regulation of the Oxphos pathway, and a

225   parallel enrichment in the glycolytic pathway. Consistent with retarded cell developmental

226   maturation is the significant increase in EC1 and EC2 early enterocyte populations with

227   homozygous *Ppargc1a* inactivation (**Fig 2D**).

228   **Rapidity and reversibility of dietary alterations:**   scRNAseq analyzed intestinal epithelial

229   cells of mice fed AIN76A for 3 months (**Fig 3A**, Arm 1), switched to NWD1 for 4 days (Arm 2),

230   and the 4d NWD1 mice switched back again to AIN76A for 4 days (Arm 3). Stem, replicating,

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231   dividing cells and all lineages were identified, with no significant changes in cell distribution for

232   clusters among the 3 arms in these short time periods (**Fig S5A,B**), and cell trajectory confirmed

233   by expression of genes known to be localized to different positions (**Fig S4B**). For the stem cell

234   cluster, 4 days of NWD1 altered 30 pathways with high significance ($P=10^{-3}$ to 10^{-43}) including

235   repression (negative Z-score) of the TCA cycle and Oxphos ($P=10^{-6}$ and 10^{-43}, respectively; **Fig**

236   **3B,C**). 19 of the 30 pathways reverted to control values in 4d NWD1 mice switched back to

237   AIN76A for 4 days. This included the TCA cycle pathway, down regulated by 4d of NWD1

238   ($P=10^{-6}$), then up-regulated when switched back to AIN76A ((**Fig 3B,C**). Expression of 8 genes

239   encoding TCA cycle enzymes were down-regulated by 4d NWD1 and reversed by 4d switch-

240   back to AIN76A (**Fig 3D**), accounting for the highly significant GSEA for dietary shifts (**Fig**

241   **3B.C**). 4d of NWD1 decreased expression of 51 Oxphos pathway genes (**Fig 3E**), consistent

242   with the highly significant negative GSEA ($P =10^{-43}$), **Fig 3B,C**), but only 15 reversed on AIN76A

243   switch back (**Fig 3E**), reflecting the absence of reversal by GSEA (**Fig 3C**). Therefore, within 4

244   days of shift to NWD1, stem cells at the crypt base underwent significant mitochondrial re-

245   programming, in large part, but not entirely, reversed within 4 days of shift back to control diet.

246   <u>**NWD1 mobilization of Bmi1+ cells:**</u> NWD1 expands the mouse intestinal proliferative cell

247   compartment (30,31), recapitulating expansion in humans at elevated CRC risk (32-35),

248   consistent with NWD1 induced lineage tracing from Bmi1+ cells above the crypt base that

249   persists for months as long as mice are fed NWD1 (6,8). Moreover, in targeting *Apc* inactivation

250   to Bmi1+ cells, tumor development increased when the mice were fed NWD1, paralleling the

251   NWD1 induction of lineage tracing, and hence stem cell-like functions, in these cells (4,6).

252   Following injury to Lgr5^{hi} ISCs, stem cell functions can be acquired by dedifferentiation of

253   cells above the crypt base (1,2). We have emphasized this plasticity also characterizes

254   adaptation of cells to their nutritional environment (4-6). To dissect this in much greater depth,

255   scRNAseq data were generated for Bmi1+ cells and their marked epithelial cell progeny isolated

256   from *Bmi1$^{cre-ERT2}$, Rosa26^{tom}* mice fed diets for 3 months from weaning (Tom+, Epcam+,

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257   CD45neg cells). The Bmi1 marker was activated at 3 months by a single Tam injection, each

258   mouse continuing on its diet for 3 or 66-70 days before sacrifice to analyze the cells within 3

259   days of marking, or after 2 months of their accumulation in the mucosa (**Fig 4A; S6A,B**)*.* Stem,

260   replicating, dividing, and all cell lineages were identified with similar cell cluster distributions

261   among cell types regardless of diet (**Fig 4B; Fig S6B,C**). Therefore, despite NWD1 repression

262   of Lgr5^{hi} stem cell functions, the mucosa was maintained. As above, predicted developmental

263   trajectory of Bmi1+ cells was confirmed by expression of markers that identify cells at different

264   positions along the crypt-luminal axis (50) (**Fig S4C**).

265   Expression of the stem cell transcription factor Ascl2 drives dedifferentiation of Bmi1+ cells

266   to replace damaged Lgr5^{hi} cells (1,2). Regardless of diet or time post Bmi1+ cell marking, *Ascl2*

267   was expressed in the Bmi1+ Stem1 and 2, Replicating and Dividing cells in trajectory analysis of

268   the data (**Fig 4B,** blue arrows, quantified in **Fig S6D)**. A small number of Tuft and goblet cells

269   also expressed *Ascl2* where goblet and enteroendocrine lineages diverged (**Fig 4B,** orange

270   arrows, and **Fig S6D**).

271   Among the 4 progenitor cell compartments, mean *Ascl2* expression per cell was highest in

272   Stem2 (**Fig 4C**). *Ascl2* expression level across each individual cell was low per cell for Stem 1,

273   Replicating and Dividing cells, with no significant difference by diet or length of time post Tam

274   cell marking (**Fig 4D**). In contrast, for Stem 2, expression per cell was heterogeneous, with a

275   subset of Stem2 cells expressing elevated *Ascl2* that accumulated longer term (**Red arrow**, **Fig**

276   **4D**). This pattern was highly significant by a likelihood ratio test (*P=0.01*, by a negative binomial

277   regression-negative binomial mixed effect model, with regression and fit using R functions

278   glm.nb and glmer.nb, respectively (53), assuming each cell as independent, and number of

279   *Ascl2* reads for each cell as response). Post-hoc analysis by a similar negative binomial

280   regression showed NWD1-LT differed significantly from the other 3 groups in *Ascl2* expression

281   (*P=0.002*). Finally, potential that the difference between NWD1-LT and the other groups was

282   random was tested by a negative binomial mixed effect model with mice per dietary group

283   treated as random effects; the likelihood ratio test was *P = 0.11*, indicating the alternate

284   hypothesis that effects were random is false.

285   *In situ* hybridization localized *Bmi+*, *Ascl2^{hi}* expressing cells confined to the crypt base in

286   AIN76A mice, but in NWD1 mice, elevated *Ascl2* was in Bmi1+ cells above the crypt base (**Fig**

287   **4E**), as in mice after acute Lgr5^{hi} cell damage (1,2), although in response to NWD1, these cells

288   are further above the crypt (Discussion).

289   **Remodeling of the mucosa by the mobilized Bmi1+ cells:** In the validated developmental

290   trajectory of Bmi+ derived cells, there are 2 early trajectory branch points among progenitor cell

291   populations (**Fig 5A**, red arrows 1 and 2). At branch point "1", Stem1 cells give rise to

292   enteroendocrine, goblet and Paneth cell secretory lineages. Data from libraries from different

293   mice gave identical results, and there were no significant changes in cell type representation by

294   diet at Branch 1 either shorter or longer term after Bmi1+ cell marking (**Fig 5B**). However, at

295   branch point 2 there were highly significant differences: in Bmi1+ cells shorter term after

296   marking (**Fig 5C**, **top**), cells from AIN76A control fed mice were equally divided between R and

297   Div cells, but in NWD1 fed mice, all cells remained in R with none in the Div cell compartment, a

298   highly significant dietary linked difference (*P=0.001*), with replicate identical patterns from

299   independent mice. This became more pronounced in Bmi1+ cells that accumulated longer term

300   (**Fig 5C, bottom**), with nearly all cells at this branch point 2 of AIN76A fed mice Div cells, but in

301   contrast, for NWD1 mice, 100% of the cells still had a transcriptional profile of R cells.

302   Independent mouse replicates were again identical, and the dietary differences highly significant

303   (*P=001*). This novel dietary effect on suppressing developmental progression of cells in their

304   trajectory along the crypt-villus axis was also reported in mice in which genetic manipulation of

305   mitochondrial structure down-regulated stem cell function (52)and in human IBD patients (39).

306   Notably, R cluster cells at branch point 2 longer term after marking express much higher *Ascl2*

307   levels (**Fig 5D**, *P=10^{-8}*) similar to the *Bmi1+, Ascl2^{hi}* cells that accumulate above the crypt base

308   in NWD1 mice (**Fig 4E**). and the Stem2 subset in NWD1 that express higher *Ascl2* in Bmi1+

309   cells that accumulate longer term after marking (**Fig 4D**).

310   **NWD1 cell reprogramming is pro-inflammatory and adaptive:** Although Bmi1+ marked cells

311   give rise to all lineages at equivalent levels in NWD1 and AIN76A fed mice, NWD1 altered gene

312   expression profiles in all Bmi1+ cell populations both long and short term after marking. This is

313   most pronounced in enterocytes, especially in EC7 enterocytes accumulating longer term (**Fig**

314   **6A**). EC7 is predicted to be the most mature enterocyte cell population, validated by markers

315   that identify villus tip cells ((50), **Fig S4C**).

316   The pathway most enriched in Bmi1+ marked EC7 cells accumulating longer term was

317   antigen processing and presentation (**Fig 6B**; NES 1.75, *P=0.009*). CD74, a key gene in this

318   pathway, is expressed in epithelial cells (54-63), mediating interaction of Lgr5^{hi} ISCs and their

319   immune environment (63). The fraction of cells expressing CD74 was highly enriched across all

320   cell clusters in longer term marked Bmi1+ cells that accumulated with time (**Fig 6C**), paralleled

321   by elevated expression per cell, with much higher expression in the EC7 mature enterocytes

322   compared to all other clusters (**Fig 6D**, *P=10^{-25}*, by negative binomial regression analysis).

323   CD74 interacts with proteins of the major histo-compatability II (MHCII) complex to process and

324   present cell surface antigens (58), and MHCII complex genes were also upregulated most

325   significantly in EC7 cells (**Fig S7A**). The greatest expression of the CD74 and MHCII complex

326   genes in EC7 cells is consistent with expression of these genes predominantly in cells in the

327   upper third of villi (64), suggesting a unique role in these differentiated cells.

328   Cell surface expression of CD74 characterizes dysplastic cells of the intestinal epithelium,

329   and cells of human Crohn's, Ulcerative, and Amebic colitis (60,65,66), chronic inflammation in

330   each elevating CRC risk. NWD1 is also pro-inflammatory, elevating serum levels of

331   inflammatory cytokines (26), and there was a 2-3 fold increase in cells expressing CD3, a pan T

332   cell marker, in 3 month NWD1 compared to AIN76A mice, significant when quantified as CD3

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333 cells/villus (*P=0.008*), or percent area/villus epithelial column expressing CD3 (*P=0.015*) (**Fig**

334 **S7B,C,D)** .

335   The second most highly enriched pathway induced by NWD1 in EC7 cells is "Intestinal

336 Immune Network/IgA production" (**Fig 6B;** NES=1.50, *P=0.027*). IgA is abundant in the

337 intestinal and colonic mucosa, traversing the lamina propria to form an SigA complex interacting

338 with the myeloid cell FcαRI receptor, which is pro-inflammatory. NWD1 also elevated F/480+

339 myeloid cells (*P=0.01*; **Fig. S6 E,F**), also characterizing pro-tumorigenic IBD (67).

340   In EC7 cells, we also identified alteration of multiple metabolic pathways, including *Ppar*

341 signaling, cholesterol metabolism, and fat digestion/absorption (**Fig. 6B**), adaptive responses to

342 NWD1 increased fat (25%). In the independent scRNAseq data of the rapid dietary cross-over

343 experiment (**Fig 3A**), fatty acid metabolism was elevated in all cell clusters by the 4-day switch

344 to NWD1 from AIN76A, and except for Goblet2 and EE2, reverted for the clusters within 4 days

345 of switch back to AIN76A (**Fig 6E**), consistent with rapid shift of metabolic pathways (**Fig 3B**).

346   Another adaptive response to NWD1 was increased number of enterocytes expressing

347 *Trpv6* in Bmi1+ marked cells, especially longer term (**Fig 6G**). *Trpv6* encodes the enterocyte

348 brush border calcium channel mediating calcium uptake under low dietary calcium conditions

349 (68). Therefore, elevated *Trpv6* expression explains how NWD1 mice maintain serum calcium

350 levels (26), despite lower calcium and vitamin D$_3$ in NWD1. Single cell ATACseq data were

351 generated from the epithelial cells of replicate mice fed AIN76A or NWD1 for 4 months. These

352 data revealed no areas of open chromatin structure for either *Cp4a10* in enterocytes, a gene

353 common to multiple pathways of fat metabolism, nor for the *Trpv6* gene, but major opening of

354 chromatin structure at and upstream of the promoter in replicate libraries for NWD1 fed mice

355 (**Fig 6F, H**), demonstrating classic epigenetic transcriptional activation of these genes generate

356 the adaptive dietary response.

357   *Ppargc1a* was down regulated specifically in stem cells at the crypt base, altering

358 mitochondrial structure that suppressed canonical Lg5^{hi} ISCs, recruiting alternates cells to

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359   maintain the mucosa. The scRNAseq had established Stem 1 harbored the Lgr5^{hi} ISCs at the

360   crypt base, consistent with subsequent trajectory analysis identifying Stem1 at initiation of

361   maturation of all cell types/lineages (**Fig 4B**). Of the 13 clusters identified in the scATACseq

362   data, the cluster aligning with Stem 1 was identified using the Signace *"Reference Mapping"*

363   function (NY Genome Center) (**Fig 7A**). The scATACseq data showed the number of these

364   cells in this stem cell cluster decreased 50% in NWD1 mice compared to AIN76A (**Fig 7B,**

365   *P=0.009*), independently confirming NWD1 suppression of Lgr5^{hi} cells. In contrast to the data

366   for *Cpt4a10* and *Trpv6* (**Fig. 6F,H**), the position and magnitude of open chromatin regions at the

367   promotor of the *Ppargc1a* in the Stem 1 cluster (designated P) is similar for all mice (**Fig 7C**).

368   However, in the region of *Ppargc1a* delineated by the boxed region, expanded in **Fig. 7D**, "a"

369   and "b" are areas of open chromatin in AIN76A mice absent or substantially reduced in NWD1

370   mice. This was quantified, showing significant reduction in signal in these regions when

371   normalized by the strong, relatively consistent signal for all samples, at the promotor (**Fig 7E**).

372   These regions of reduced accessibility in NWD1 mice correspond to the position of a strong

373   enhancer in the gene with a significant role in regulating gene expression in intestinal epithelial

374   cells (**Fig 7C,D**; *Enhanceratlas 2.0*). Thus, the scATACseq data confirm NWD1 down-regulated

375   expression of *Ppargc1a* expression, and establish this is epigenetic, but likely by a more

376   complex mechanism than altering accessibility of sites at or upstream of the promoter.

377

Discussion:

379   Adjusting multiple nutrient exposures in the mouse to mimic levels elevating risk for human

380   sporadic colon tumors causes rapid, reversible and hence dynamic adaptation of stem cells and

381   lineages through epigenetic mechanisms that also alter pathways establishing a pro-tumorigenic

382   environment. The data provide seven fundamental insights into dietary mechanisms of tumor

383   risk, many paralleling pathogenic mechanisms of human inflammatory bowel disease: 1) low-

384   level chronic inflammation that is pro-tumorigenic; 2) NWD1 reduced *Ppargc1a* expression in

385   Lgr5hi cells through epigenetic alteration of the gene, lower expression also mediating

386   repression of Lgr5hi cell stem cell functions in IBD (69), and down regulation of the encoded

387   protein, Pgc1a, that also exacerbates human IBD (38), in both cases altering mitochondrial

388   structure, metabolically reprogramming the cells; 3) feeding NWD1 or direct genetic inactivation

389   of the *Ppargc1a* gene in Lgr5hi cells retarded developmental maturation of cells as they migrated

390   along the crypt-lumen axis, similar to retarded maturation of cell differentiation in human IBD

391   along this axis also recently determined from trajectory analysis of single cell data (39); 4)

392   NWD1 induced greatest perturbation of expression profile in mature enterocytes at the villus tip,

393   where the most prominent change is elevation in the interacting CD74-MHCII pathways of

394   antigen presentation and processing. This parallels highest expression of these pathways in

395   upper villi of the human small intestine (64) and that in human IBD, upper crypt colonocytes

396   exhibit greatest increase in this pathway (37). Further, NWD1 increased pro-inflammatory

397   cytokines (26), and CD3 and myeloid cell mucosal infiltration, paralleling elevation of the

398   CD74/MHCII pathways in human Crohn's, Ulcerative, and Amebic colitis (60,65,66), confirmed

399   by single cell analysis of human IBD tissue (37), and also in H.pylori infected gastric tissue

400   (57,70), all chronic inflammatory states increasing risk for human tumor development; 5) NWD1

401   induces high ectopic expression of Lyz and other Paneth cell markers in mouse colon (29),

402   similar to Lyz elevation in human IBD tissue (37) and single cell RNAseq human data document

403   expression of these markers in cells of the human mucosa that are not Paneth cells (71). Rapid

404   alteration of Paneth cell markers in response to a 60% fat diet has also recently been reported

405   (7). 6) In NWD1 fed mice, extensive remodeling of stem cells and lineages reflects alterations

406   in cell reprogramming in both the involved and uninvolved mucosa in human IBD (37,39), and in

407   the earliest benign human tumors (72), indicating remodeling is initiated early in the mucosa at

408   dietary risk; 7) Establishment that the nutritional environment can shift which stem cells function

409   in mucosal maintenance parallels a recent report that human colon tumors consist of both Lgr5

15

410   positive and negative stem cells with the balance determined by the mutational signature and

411   environmental signals from other cell types or drugs (36). The multiple parallels between NWD1

412   induced alterations and those in human IBD suggest that the dietary and IBD remodeling, and

413   increased tumor risk, may share common mechanisms of pathogenesis.

414   The substantially increased CD74/MHCII pathways in NWD1 fed mice, similar to that in

415   involved and uninvolved mucosa in human IBD, contrasts with an important report that a 60% fat

416   diet repressed these pathways in mouse Lgr5^{hi} ISCs, suggested to be pro-tumorigenic by

417   dampening immune surveillance (73). Several factors may contribute to this difference, including

418   major differences in dietary formulation; 60% dietary fat to induce mouse obesity exceeds fat

419   intake in even obese humans, and induces metabolic alterations differing from those induced by

420   dietary fat levels more common in humans (15,16). The 60% fat diet, as do all commonly used

421   rodent diets, also includes very high levels of vitamin D exceeding the mean level in humans by

422   200-300%, and well above even the 1% of the population at very highest levels. This is

423   fundamental since robust expression of the vitamin D receptor is a core component of the Lgr5^{hi}

424   stem cell signature, and we showed and extensively discussed the essential role of vitamin D in

425   Lgr5^{hi} stem cell function, as it is in hair follicle and other stem cells (8,9,74). Further, as discussed

426   in detail (4,6,8,9), the much lower vitamin D levels in humans likely contributes to the fact that it

427   takes 50 fold longer for CBC stem cells in humans to reach clonality than in the mouse (1,75,76).

428   Thus, the 60% fat diet, and other commonly used mouse diets, establish a nutritional environment

429   strongly supporting Lgr5^{hi} ISC functions, but this environment necessary for robust function of

430   Lgr5^{hi} stem cells is simply not present in nearly all humans.

431   NWD1 remodeling of stem cells and lineages includes epigenetic regulation of pathways

432   mediating the rapid and reversible adaptation of the mucosa to its nutritional environment. This

433   included upregulated *Trpv6* expression in enterocytes, the gut calcium transporter maintaining

434   calcium homeostasis under low calcium conditions, explaining how, despite lower calcium in

435   NWD1, serum calcium level is maintained with no loss of bone mineral density (26). A second

16

436   example is rapid and reversible alteration of fat metabolism pathways in response to the 25% fat

437   in NWD1, as recently reported for a 60% fat diet (7). For both NWD1 examples, elevated

438   expression paralleled opening of chromatin configuration at the gene promoter, independently

439   confirming response to NWD1 and demonstrating epigenetic regulation.

440   The significance of the metabolic reprogramming of Lgr5^{hi} stem cells by NWD1 is emphasized

441   by necessity of Oxphos for mouse Lgr5^{hi} and Drosophila intestinal stem cells to function efficiently

442   as stem cells (40-42), for hematopoietic stem cell functions (43), for embryonic stem cell

443   pluripotency (44), and a major role of mitochondrial function in determining whether stem cells

444   self-renew or differentiate (51). The repression of Lgr5^{hi} cells as stem cells recruits alternate

445   *Bmi1+, Ascl2^{hi}* cells above the crypt base for mucosal maintenance, as in response to acute

446   damage. *Ascl2* encodes a stem cell transcription factor essential for Bmi1+ cell de-differentiation

447   in response to Lgr5^{hi} cell damage (1,2). *Ascl2* is regulated by Wnt signaling (77), elevated by

448   NWD1 throughout mouse intestinal villi and colonic cells (27-29). However, there are likely

449   different roles of *Bmi1+, Ascl2^{hi}* cells in NWD1 versus damage induced plasticity. Acute damage

450   purges Lgr5^{hi} ISCs mobilizing *Ascl2^{hi}* cells to migrate into the crypt to restore crypt organization.

451   However, limited crypt space establishes competition among crypt Lgr5^{hi} cells (1), and single cell

452   laser ablation of crypt base cells triggers division and reorganization of remaining stem cells,

453   confirming importance of physical space in regulating stem cell dynamics (78). In contrast, crypt

454   space may be unavailable with chronic NWD1 feeding. Further, cells migrating into the niche

455   would still be repressed by NWD1, and reduced Lgr5^{hi} cell lineage tracing from the crypt base,

456   and Oxphos and TCA pathway repression, persist to at least 1 year of feeding NWD1 (8) (**Fig S1,**

457   **2**), with Bmi1+ cells above the crypt base lineage tracing for months in mice continuously fed

458   NWD1 (6).

459   Tumors reflect growth advantage of transformed cells (1), but this is very rare: lower-risk

460   individuals develop no sporadic tumors, and those at higher risk only 1-2 tumors despite billions

461   of mucosal cell divisions over decades, suggesting mechanisms establishing risk are subtle and

17

462 complex. Therefore, it is fundamental that the nutritional environment, through metabolic

463 reprogramming of stem cells, dynamically and continually sculpts the playing field on which

464 intestinal stem cell competition takes place, with significant influence in determining outcome.

465

466 **Experimental Procedures:**

467 **Mice:** Mice, on a C57Bl/6J background, were provided food and water *ad libitum* in a barrier

468 facility at the Albert Einstein College of Medicine. For breeding, strains were fed a chow diet

469 (Picolab 5058, Fisher Feed, Branchburg, NJ). Appropriate genotypes were randomized to

470 purified diets (AIN76A, D10011; NWD1, D16378C; Research Diets Inc, New Brunswick, NJ).

471 Mice of both genders were used (a total of 25 male and 20 female mice). Tamoxifen in corn oil

472 was a single injection (Sigma, T5648, 100μl, 1mg/μl). Experiments were approved by the Albert

473 Einstein Institutional Animal Care and Use Committee. All authors had access to the study

474 data.

475 **Isolation of intestinal epithelial cells:** On sacrifice, excised intestines were opened

476 longitudinally, rinsed with cold saline, crypts isolated, single cell suspensions prepared, and

477 depending on the experiment stained with EpCAM-FITC+ (Miltenyi Biotec, Clone caa7-9G8, Cat

478 No. 130-123-674), CD45-PerCP negative (Miltenyi Biotec, Clone 30F11, Cat No. 130-123-879)

479 sorted by FACs and collected on a MoFlo instrument as described (6)

480 **Bulk RNAseq analysis:** construction of bulk RNAseq libraries, sequencing and data analysis

481 were as described (6).

482 **Single cell RNAseq:** Single cell RNAseq (scRNAseq) libraries were constructed by the Albert

483 Einstein Genomics Core using the 3' kit protocols of 10X Genomics (San Francisco, CA) with

484 approximately 10,000 single FACS collected cells from each mouse loaded onto a Chromium

485 Chip B microfluidic apparatus. Library quality was verified by size analysis (BioAnalyzer; Agilent,

486 Santa Clara, CA) and after passing quality control assays, multiple libraries mixed and

487 sequenced by HiSeq PE150 or NovaSeq PE150 using pair-end sequencing with a readout

488 length of 150 bases (Novogene; Sacramento, California), and the data assigned to individual

489 cells using the sample indexes.

490   For sequence alignment and subsequent analysis, output Illumina base call files were

491 converted to FASTQ files (bcl2fastq), aligned to the mouse mm10 genome v1.2.0 and converted

492 to count matrices (Cell Ranger software v3.0.2). Force-cell parameter was used, with 5000-

493 8000 individual cells identified for each sample, and unique molecular identifiers (UMI) identified

494 to remove PCR duplicates. Quality control and downstream analyses were done in R v4.0.2,

495 using Seurat package v3.2.2 (79). To discard doublets or dead cells, cells with gene counts

496 <200 or >5000, or a mitochondrial gene ratio >20%, were filtered out. Cells from different

497 samples were integrated using Seurat FindIntegrationAnchors and IntegrateData functions and

498 clusters identified from each integrated data set using Seurat FindClusters. This calculates k-

499 nearest neighbors by Principle Component Analysis, constructs a shared nearest neighbor

500 graph, and then optimizes a modularity function (Louvain algorithm) to determine cell clusters.

501 Cell clusters were identified as cell intestinal cell types using established cell-type markers and

502 cluster identification using Seurat FindMarkers function. Differential gene expression compared

503 samples among experimental groups: initial criteria were an expression change of ≥1.5 fold with

504 associated adjusted P-value of <0.01 in group comparison (Seurat FindMarkers function).

505 Pathway analysis was performed on differentially expressed genes using clusterProfiler R

506 package v3.16.1 and the KEGG pathway database (KEGG.db R package v3.2.4); gene set

507 enrichment analysis (GSEA) used the fgsea R package v1.14.0, the MSigDB (v5.2) hallmark

508 gene set and the KEGG pathway database. Pathways at *P <0.05* were considered statistically

509 significant. Trajectory analysis used Monocle3 R package v0.2.3.3 (80) with cluster information

510 and cell-type identifications migrated from Seurat, the integrated dataset divided among the

511 experimental conditions, and trajectories generated for experimental group.

512   RNAseq data have been deposited in the NCBI Gene Expression Omnibus (GEO)

513 database: bulk RNAseq (Fig 1) - GSE186811; scRNAseq (Fig 2, *Ppargc1a* inactivation) -

514   GSE188339; scRNAseq (Fig 3, rapid dietary crossover) - GSE188577; scRNAseq for Bmi1+

515   cells (Fig 4.5) - GSE188338.

516   *In situ* analyses: Sections of mouse intestinal swiss rolls fixed in 10% formalin were de-waxed

517   and rehydrated. Reagents were from Advanced Cell Diagnostics (Newark, CA). Endogenous

518   peroxidases were quenched and heat induced epitope retrieval done utilizing the

519   RNAscope Multiplex Fluorescent Reagent Kit v2 (Cat. No. 323100) and the RNAscope

520   Multiplex Fluorescent Assay v2 protocol followed. RNAscope target probes for *Bmi1* and *Ascl2*

521   Cat No. 466021, 412211, respectively) were hybridized to sections and amplified using the

522   HybEZ Hybridization System (Cat No. 310010). Secondary TSA fluorophores from Akoya

523   Biosciences were Opal 620 (channel 1, Cat No. FP1495001KT) and Opal 570 (channel 2,

524   FP1488001KT), sections counterstained with Dapi and visualized using a Leica SP8 confocal

525   microscope (20X magnification).

526   **Single cell ATACseq:** Total Epcam+, CD45 negative epithelial cells from the small intestine of

527   mice were FACs isolated as for scRNAseq. Nuclei were isolated following the 10X Genomics

528   protocol, single cell ATACseq libraries constructed using the 10X Geomics single cell ATACseq

529   Chromium reagents and protocol, and sequenced as for scRNAseq.

530   **Immunohistochemistry:** Swiss roll sections were de-waxed, rehydrated, endogenous

531   peroxidases blocked (3% hydrogen peroxide in methanol) and heat induced epitope retrieval

532   done with 10 mM Sodium citrate buffer (pH 6.0) for all antibodies. Tissues were incubated

533   overnight at 4^0C with primary CD3 antibody (Novusbio, Littleton, CO, 1:10 dilution of NB600-

534   1441). Protein block and secondary antibody for CD3 employed ImmPRESS-Alkaline

535   Phosphatase Horse Anti-Rabbit IgG Polymer Kit-Alkaline Phosphatase (Vector Laboratories,

536   Burlingame, CA, Cat No. MP-5401). F4/80 antibody (1:200, Cell Signaling, Denvers, MA, Cat

537   No. 70076, Cell Signaling, Denvers, MA) was incubated on tissue overnight at 4^0C. Secondary

538   antibody was SignalStain® Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling, Cat No.

539   8114). For Pgc1a staining, tissues were incubated overnight at 4 °C overnight with rabbit anti-

20

540 Pgc1a Ab (diluted 1:500, NBP1-04676, Novusbio) followed by biotinylated goat anti-rabbit IgG

541 (1:100, BA-1000; Vector Lab,) for 30 min. Visualization used the Vectastain Elite ABC Kit

542 (Vector Lab) and DAB Quanto kit (Thermo Fisher Scientific). Counterstaining was with

543 hematoxylin and visualization by bright field microscopy, or a CY5 filter for CD3.

544 **Electron Microscopy:** Samples were fixed with 2.5% glutaraldehyde, 2% paraformaldehyde in

545 0.1 M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide followed by 2% uranyl

546 acetate, dehydrated through a graded ethanol series and embedded in LX112 resin (LADD

547 Research Industries, Burlington VT). Ultrathin sections were cut on a Leica Ultracut UC7, stained

548 with uranyl acetate followed by lead citrate and viewed on a JEOL 1400 Plus transmission electron

549 microscope at 120kv. Images were captured at a magnification of 800X, and at 5000X for

550 quantitative measurements of mitochondrial size and cristae density.

551 **Mitochondrial membrane potential:** Crypts were dispersed into single-cell suspensions, and

552 cells incubated with MitoTracker^{TM} Red FM (Invitrogen, Cat # M22425, 25nM) for 20 min at 37

553 °C. Zombie NIR (Invitrogen, Cat # L10119) was then added to mark dead cells. Flow cytometry

554 used a Cytek Aurora Instrument (Cytek Biosciences) with analysis using Flowjo 10 software

555 (Treestar Inc.).

556 **Lineage tracing of *Ppargc1a* knock-out mice:** Induction of *cre^{ERT2}* activity was by a 100 µl

557 intraperitoneal injection of 1 mg freshly prepared Tamoxifen (TAM) in sterile corn oil. Mice were

558 sacrificed 3 days after injection, intestinal tissues fixed with 4% paraformaldehyde for 2 hours,

559 hydrated with 15% sucrose for 3 hours, and 30% sucrose overnight. Fixed tissues were

560 embedded in optimal cutting temperature (OCT) compound (Sakura), frozen on dry ice,

561 cryostat sectioned, and stained with DAPI for identification of nuclei before imaging.

562 **Image analysis:** To quantify anti-Pgc1a Ab staining, images were at 60x magnification. A

563 threshold was applied for all slides, and the area above threshold calculated and divided by total

564 area. The crypt-villus axis was divided by 20 µm increments from the base of the crypt and

565 measured up to 80 µm. For analysis of mitochondrial morphometry, crypt base columnar cells,

21

566   Paneth cells, and villus cells imaged at 5000x magnification were selected. Cristae density was

567   calculated by the sum of individual cristae divided by mitochondrial size. Mitochondrial size

568   (μm^2) and cristae area (μm^2) were manually traced using Fiji. For lineage tracing quantification,

569   the crypt-villus axis was manually traced and the threshold automatically adjusted for each

570   crypt. Red fluorescent positive area (μm^2) was measured and divided by total crypt-villus area

571   (μm^2).

572

573   **Figure legends:**

574

575   **Figure 1: Dietary impact on intestinal stem cells: A,** $Lgr5^{EGFP.cre:ER}$ mice fed AIN76A or

576   NWD1 for 3 or 12 months from weaning, or NWD1 for 3 months then switched to AIN76A for 9

577   months (cross-over); **B - D,** *Ppargc1a* expression by bulk RNAseq of Lgr5^{hi} cells of 2 different

578   mouse cohorts fed either NWD1 or AIN76A for 3 months from weaning (**B, D**), or for NWD1 or

579   AIN76 for 12 months compared to NWD1 for 3 months and then switched to AIN76A for an

580   additional 9 months (**C -** Arm 3, Fig 1A); **E, F** Pgc1a immunohistochemistry and quantitation in

581   crypts of mice fed AIN76A or NWD1 for 3 months; **G,** *Ppargc1a* expression in Lgr5^{hi} and Bmi1+

582   cells from bulk RNAseq analysis of mice fed different diets for 3 months; **H,** examples of

583   mitochondria in wild-type mice fed AIN76A or NWD1, or mice fed AIN76A and *Ppargc1a*

584   homozygously inactivated in Lgr5 cells (800X); **I,** quantitation of mitochondrial cristae density in

585   CBC, Paneth, and villus cells evaluated at 5000X magnification; **J,** FACs analysis of Lgr5^{hi} cell

586   number; **K,** Mitotracker staining of mice fed AIN76A or NWD1 for 3 months. *(*P<0.05, **0.01,*

587   ****0.001).*

588

589   **Figure 2: Genetic inactivation of *Ppargc1a* in Lgr5 cells: A,** suppressed lineage tracing from

590   Lgr5^{hi} cells by homozygous inactivation of *Ppargc1a* in $Lgr5^{cre:er-GFP}$, $Ppargc1a^{F/+ \text{ or } -/-}$, $Rosa26^{tom}$

591   mice fed AIN76A diet, 3 days post a single Tam injection; **B,** quantification of lineage tracing; **C,**

592   cluster map and cell types, N=3 independent mice for each genetic group (9 scRNAseq

593   libraries); **D**, epithelial cell distribution in clusters/lineages in wild-type mice or with heterozygous

594   or homozygous *Ppargc1a* inactivation targeted to Lgr5^{hi} cells; **E-G**, trajectory analysis from

595   scRNAseq of total intestinal epithelial cells from wild-type mice or 3 days after hetero- or

596   homozygous *Ppargc1a* inactivation: yellow arrow, Stem cell cluster; **H, I** cell type distribution at

597   branch point "a" and "b", respectively (red arrows in E-G) of wild-type, het and hom inactivation

598   of *Ppragc1a* and GSEA of pathways of *Ppargc1a$^{-/-}$* compared to wild-type mice at those branch

599   points (numbers indicate *P values*).

600

601   **Figure 3: Rapid reprogramming of cells by dietary shift: A,** mice fed AIN76A for 3 months

602   (Arm1), switched to NWD1 for 4 days (Arm 2), or then switched back to AIN76A for 4 days (Arm

603   3), total Epcam+, CD45neg epithelial cells FACs isolated and analyzed by scRNAseq; **B,**

604   pathways significantly altered by rapid dietary shifts and their negative log *P* value for

605   significance of pathway change; **C**, magnitude of change of each pathway under the different

606   dietary conditions; **D,** TCA cycle genes repressed by switching mice from AIN76A to NWD1 for

607   4 days and then elevated when mice switched back to AIN76A control diet for 4 days; **E,** altered

608   expression of each gene in the Oxphos pathway by 4 day shift from AIN76A to NWD1, and

609   response of each to subsequent switch back to AIN76A for 4 days.

610

611   **Figure 4: scRNAseq of Bmi1+ intestinal epithelial cells in response to diet: A,** Rosa26^{tom}

612   marked Epcam+, CD45neg epithelial cells FACs isolated from *Bm1$^{cre:er}$, Rosa26^{tom}* mice fed

613   AIN76A or NWD1 for 3 months, then sacrificed at 3 or 66-70 days after TAM injection to activate

614   the Rosa^{tom} marker (shorter, longer term, respectively) and cells analyzed by scRNAseq (N=2

615   for each group*); **B,** cell trajectory analysis as a function of diet and time post marking by Tam

616   injection: Blue arrows, *Ascl2* expressing cells among stem and progenitor cells. or yellow

617   arrows, in goblet and enteroendocrine cells; **C**, **D,** expression of *Ascl2* per cell in Stem1, 2,

618   Replicating and Dividing cells clusters: red arrow in **D** for Stem 2 cells is a population that

619   expressed *Ascl2* at a higher level (***statistical analysis detailed in text); **E,** *Ascl2* and *Bmi1*

620   expression by *in situ* hybridization in mice fed diets for 3 months: white dotted line demarks the

621   crypt base.

622

623   **Figure 5: A**, trajectory analysis of Bmi1+ marked cells at 3d or 66-70 days post Tam activation

624   of the Bmi1 marker, annotated with individual cell types: red arrows/numbers denote branch

625   points analyzed; **B, C** cell type distribution at branch points "1" and "2" (red arrows in A); **D**,

626   *Ascl2* expression per cell at branch point 2.

627

628   **Figure 6: NWD1 reprogramming and adaptation of cells: A,** number of differentially

629   expressed genes (>50% *and P=0.01*) in Bmi1 cell clusters of Fig S6B; **B,** Heat map of genes

630   differentially expressed by diet in EC7 cells and cell pathways enriched (GSEA) as a function of

631   diet in Bmi1+ EC7 cells longer term post marking of Bmi1+ cells (statistical analysis detailed in

632   text); **C**, fraction of cells in each cluster expressing CD74; **D**, CD74 expression per cell, in each

633   cell cluster as a function of diet and time post marking of Bmi1+ cells; **E,** GSEA analysis for

634   each cell cluster for the fatty acid metabolism pathway in the rapid dietary cross-over

635   experiment (Fig 3A) – red bars, pathway change in mice fed AIN76A for 3 months, then

636   switched to NWD1 for 4 days before sacrifice; blue bars, the mice then switched back to

637   AIN76A for 4 more days; **F**, scATACseq data for *Cyp4a10* in enterocytes; **G,** scRNAseq data

638   for *Trpv6* Bmi1+ derived cells from AIN and NWD1 fed mice 3 days or 66-70 days after cells

639   were marked (Fig 4A); **H**, scATACseq data for *Trpv6* in cells in enterocytes for mice fed either

640   AIN76A or NWD1 for 4 months from weaning .

641

642   **Figure 7: single cell ATAC seq data, stem cells: A,** clusters from the scATACseq data; **B**, per

643   cent Stem 1 cells in mice fed AIN76A or NWD1 based on the scATACseq data; **C,** scATACseq

644   data for the *Ppargc1a* gene in the Stem 1 cluster. Box delineates region of diminished peaks in

645   NWD1 compared to AIN76A fed mice, and P denotes the promoter region; **D,** boxed region in C

646   expanded, with "a" and "b" denoting areas within this region where peaks in AIN fed mice are

647   substantially diminished in NWD1 fed mice; **E**, quantification of reads in regions a and b relative

648   to reads at the promotor for mice fed the different diets; statistical analysis by a poisson

649   regression on aggregated read counts over all cells per mouse, normalized by coverage per

650   sample in the promoter region of *Ppargc1a*.

651

652   **Supplemental Figures**

653   **Figure S1: A,** number of the ~8000 expressed sequences in Lgr5^{hi} cells up or down-regulated

654   (≥50%, *P≤0.01)* at 3 or 12 months feeding NWD1 compared to AIN76A, or 3 months NWD1

655   then switched to AIN76A for 9 months (Arm 3, Fig 1); **B,** Pathways significantly altered by Gene

656   Set Enrichment Analysis of bulk RNAseq data of Lgr5^{hi} cells isolated by FACS from mice fed

657   NWD1 compared to AIN76A for either 3 or 12 months, or for mice fed NWD1 for 3 months than

658   switched back to AIN76A for 9 months, described in Fig 1A.

659

660   **Figure S2:** GSEA of the TCA cycle and Oxphos pathways in mice of the 3 Arms of Fig. 1A.

661   Inserts in each panel show the NES, *P-value* and false discovery rate (FDR).

662

663   **Figure S3: A,** Expression ratio (bulk RNAseq) of Oxphos pathway genes in Lgr5^{hi} cells of mice

664   fed NWD1 vs AIN76A for 3 months (Fig 1A) or of an independent bulk RNAseq data set from

25

665   different mouse cohorts (6) and **B**, Bulk RNAseq expression ratio of Oxphos pathways genes in

666   Lgr5^{hi} cells fed NWD1 compared to AIN76A for 12 months or in the cross over arm of Fig 1A,

667   NWD1 for 3 months switched to AIN76A for an additional 9 months (12 months total) compared

668   to mice fed NWD1 continuously for 12 months. Statistical analysis detailed in text; **C**, summary

669   of the down-regulated genes encoding subunits of each of the 5 mitochondrial electron transport

670   chain complexes.

671

672   **Figure S4**: Validation of predicted trajectory from scRNAseq data by expression of markers

673   shown to be physically localized along the crypt-villus axis (villus bottom, mid-upper villus and

674   villus tip) (50). **A**, trajectories of Fig 2, *Ppargc1a* genetic inactivation in Lgr5^{hi} cells; **B**,

675   trajectories of Fig 3, rapid dietary cross-over; **C**, trajectories of Fig 4A and 5A, Bmi1+ marked

676   cells.

677

678   **Fig S5: Rapid dietary cross-over experiment of Fig 3. A,** cluster map and cell types, N=3

679   independent mice for each dietary Arm (9 scRNAseq libraries – Fig 3A); **B**, epithelial cell type

680   distribution in clusters/lineages.

681

682   **Fig S6: scRNAseq analysis of Bmi1+ derived cells. A**, Bmi1+ derived epithelial cells from

683   mice fed AIN76A or NWD1 FACs isolated 3 or 66-70 days post marking (Fig 5);

684   **B,** cluster map of intestinal epithelial cell types/lineages – red arrow, stem cell cluster**; C,**

685   intestinal epithelial cell type/lineage distribution by diet and time post Tam marking of cells; **D,**

686   fraction of cells Ascl2 positive in each cluster by diet and time post marking.

687

688   **Fig S7: Inflammatory response to NWD1: A,** expression of 5 genes encoding proteins of the

689   MHCII pathway across cell type/lineages in relation to diet and shorter term (ST= 3d) or longer

690   term (LT=66-70d) post Tam marking of Bmi1+ cells; **B**, immunohistochemical detection of CD3+

691   cells in AIN76A and NWD1 fed mice; **C, D,** quantitation of CD3+ cells in relation to diet by

692   number of CD3+/villus (**C**) or area of red fluorescent staining in the mucosa (**D**), N=3 mice per

693   dietary group; **E**, F480+ cells in AIN76A and NWD1 fed mice; **F**, staining intensity per villus, N=3

694   mice per dietary group.

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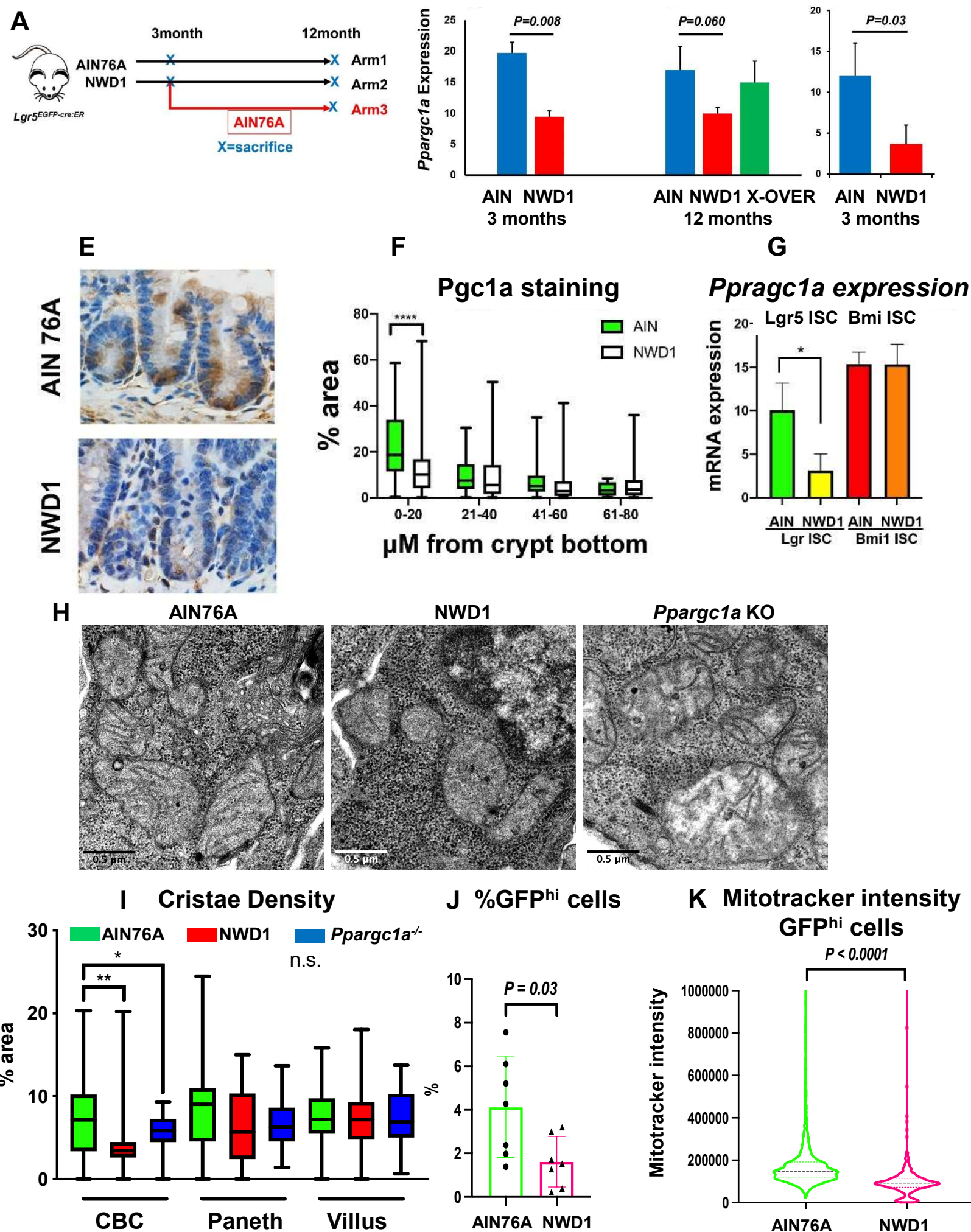
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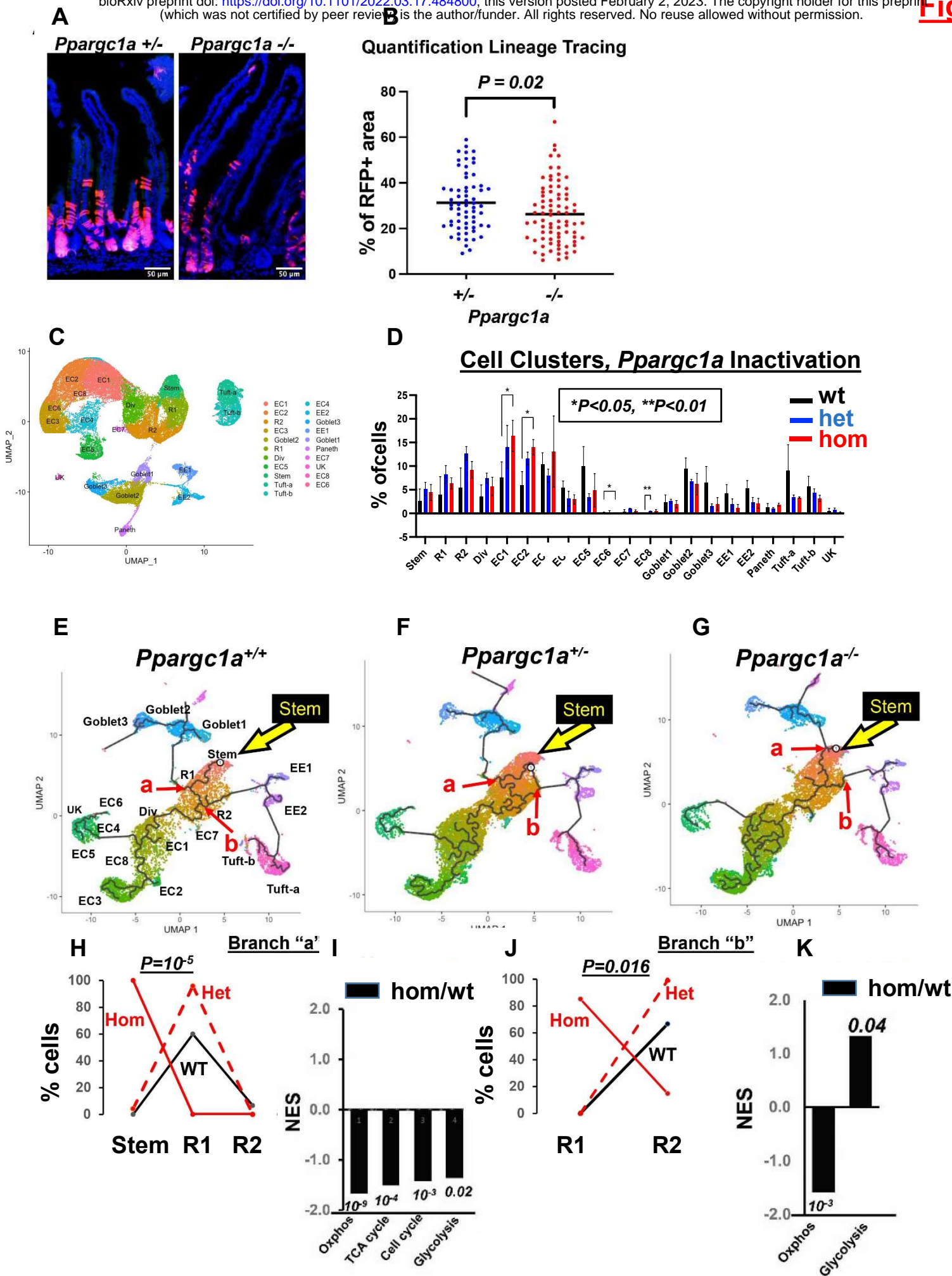
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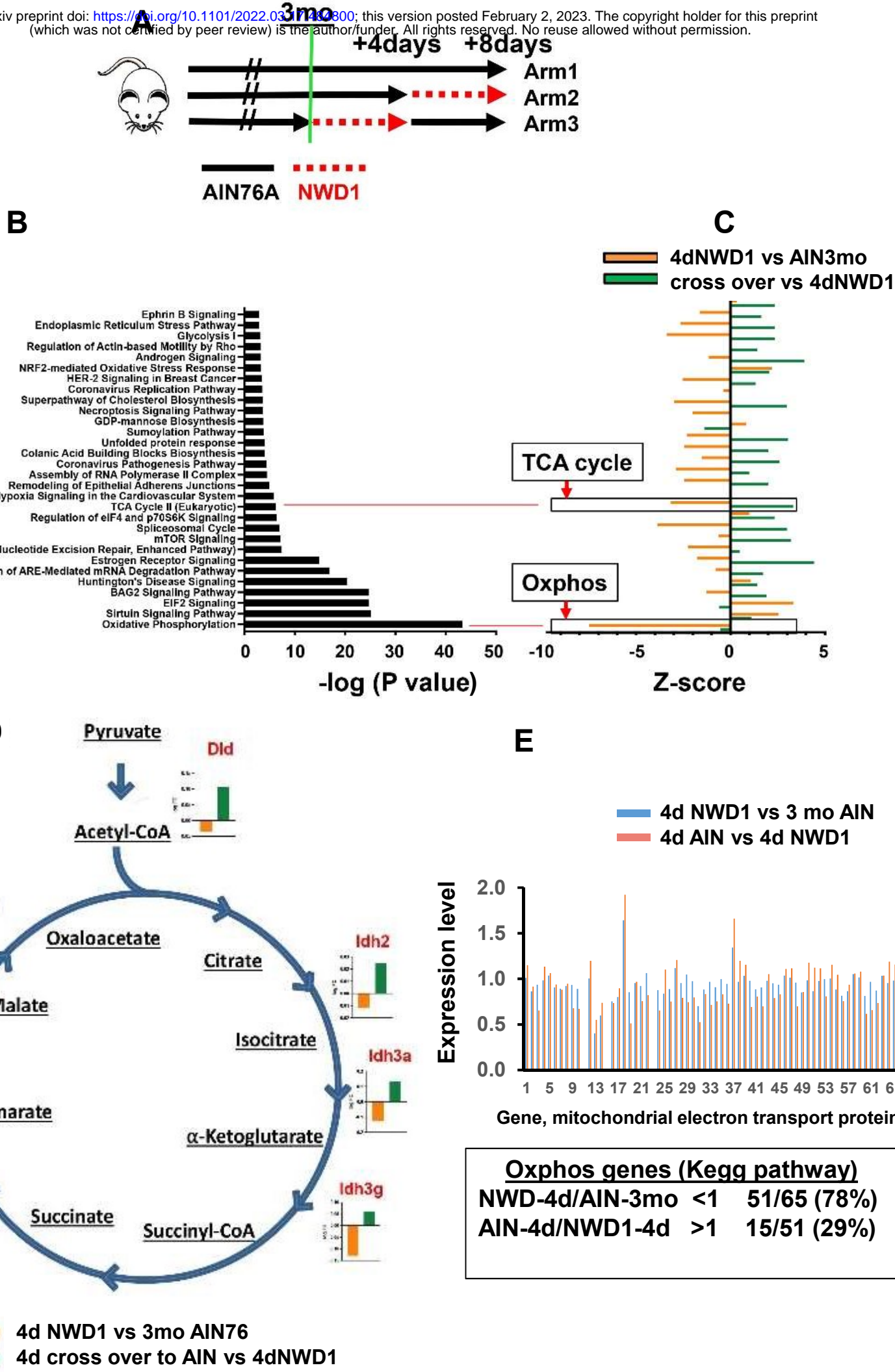883   **2001**;2(11):1061-6 doi 10.1038/ni720.

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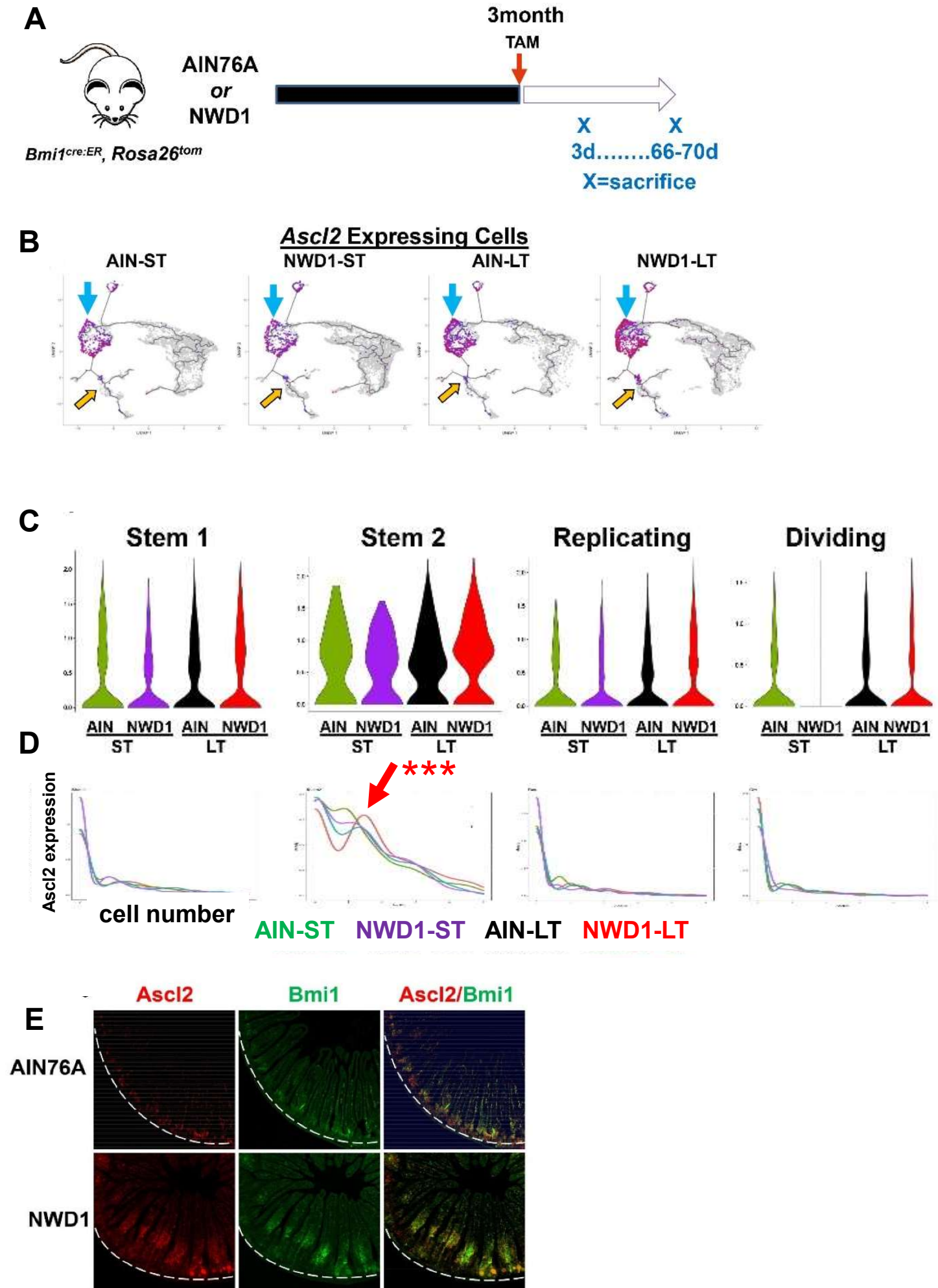
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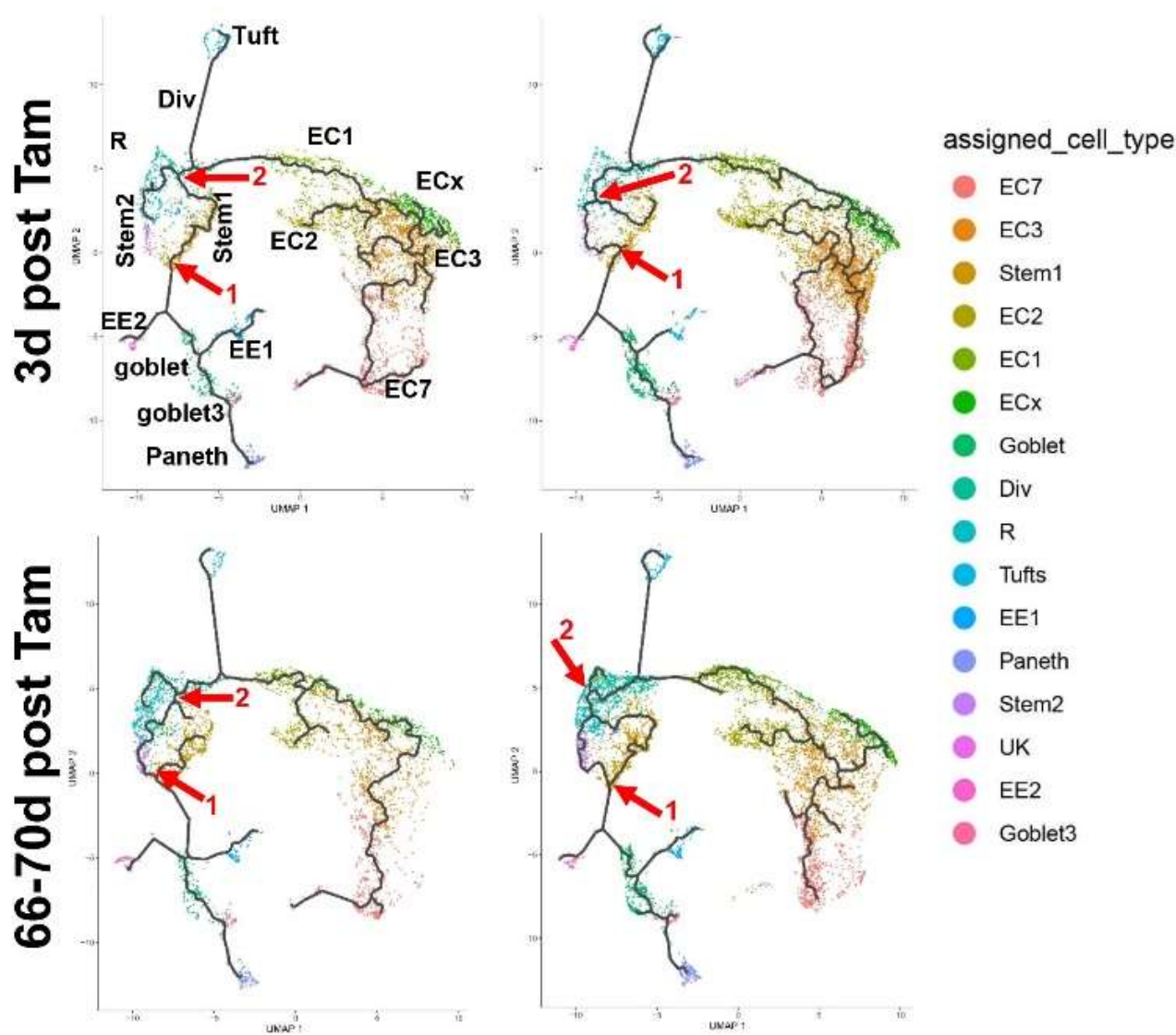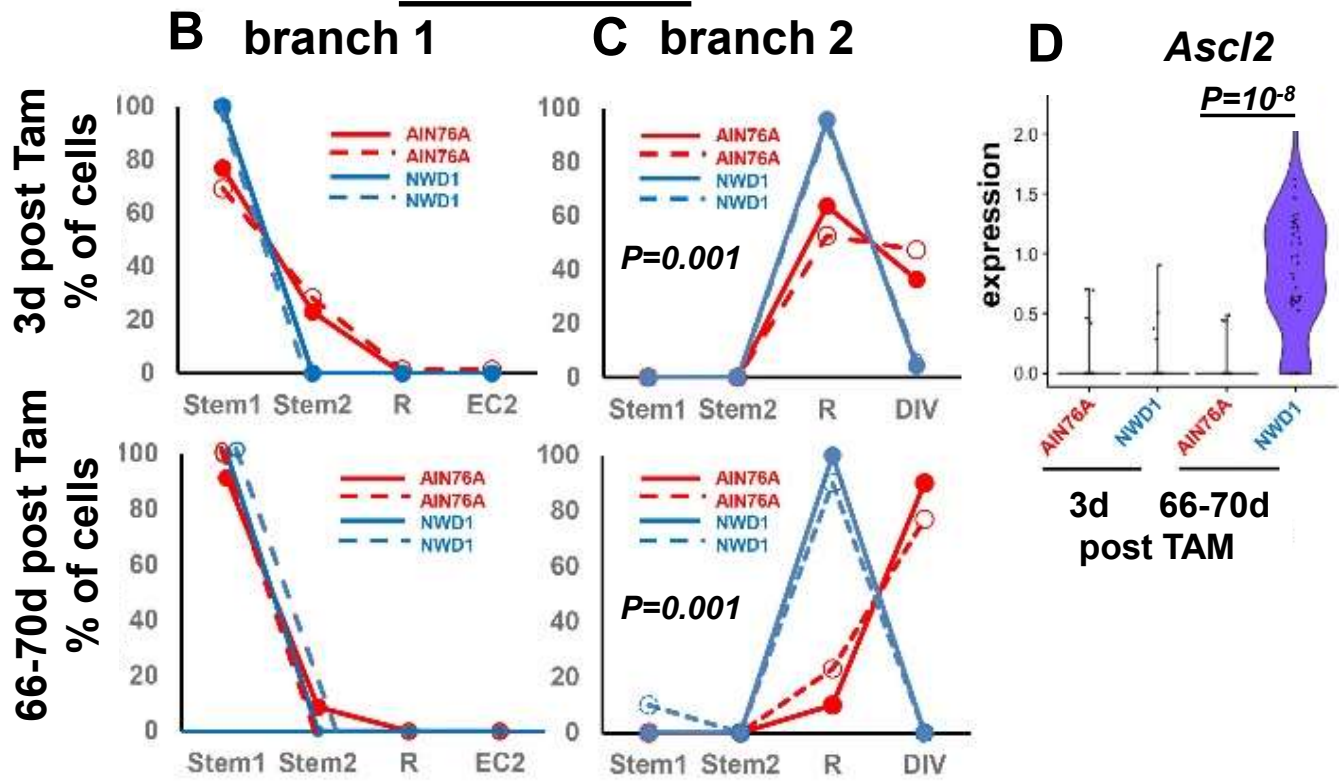
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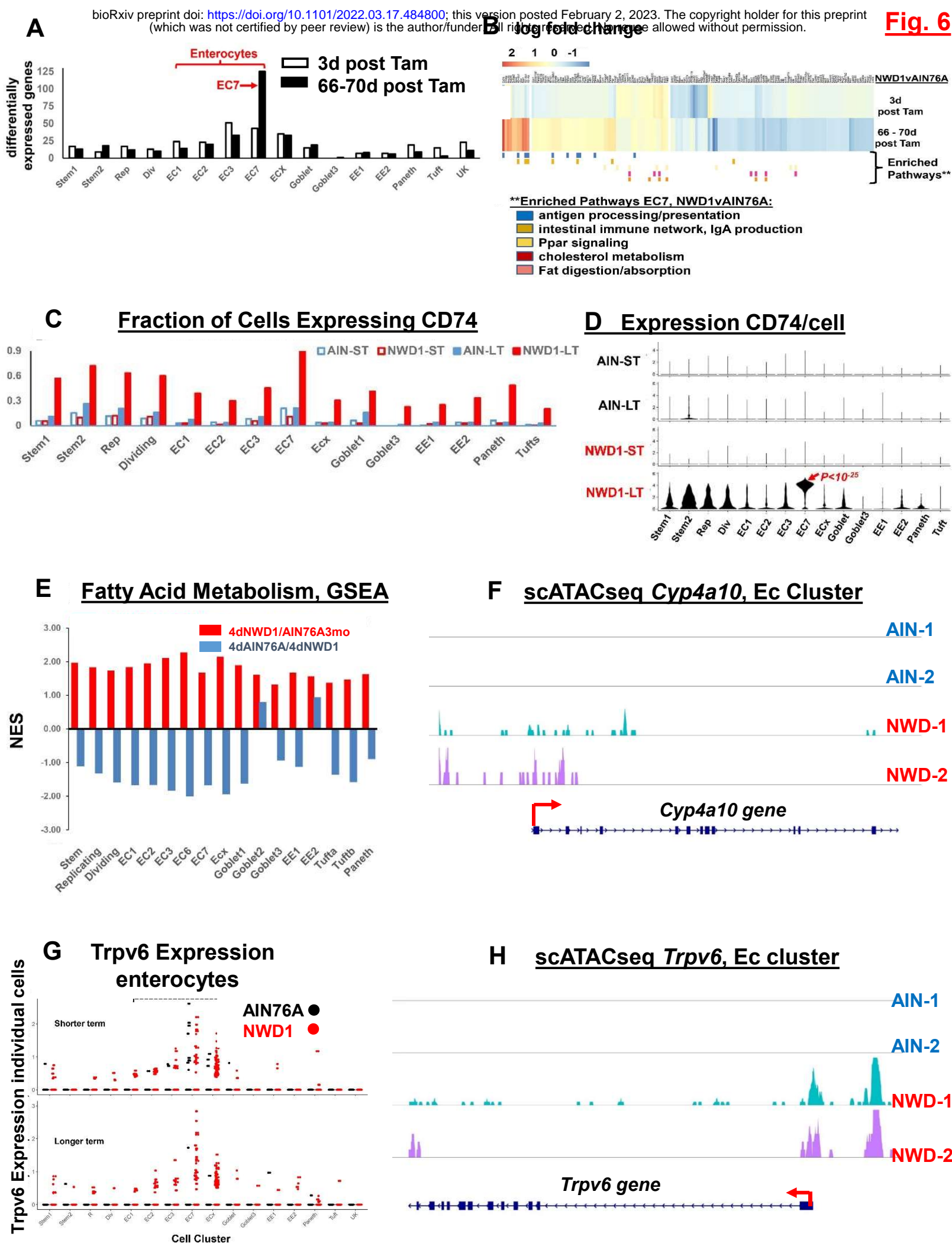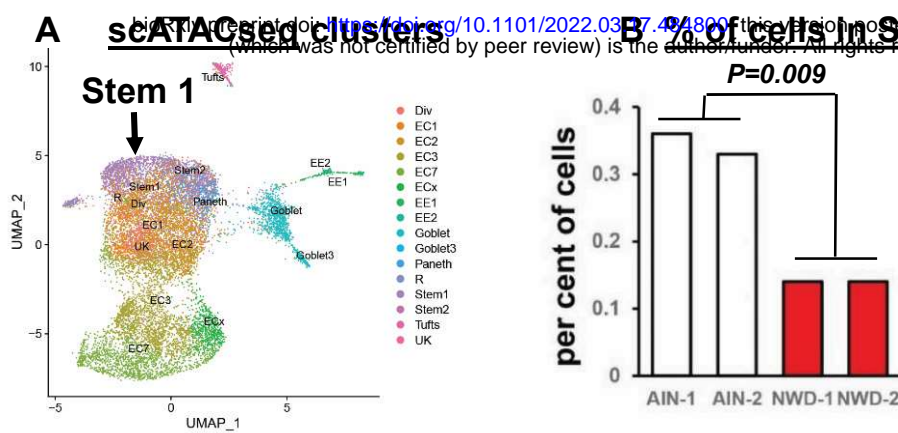
Fig. 1

Fig. 2

Fig. 4

Fig 5

A

AIN76A NWD1

3d post Tam

66-70d post Tam

assigned_cell_type
- EC7
- EC3
- Stem1
- EC2
- EC1
- ECx
- Goblet
- Div
- R
- Tufts
- EE1
- Paneth
- Stem2
- UK
- EE2
- Goblet3

Bmi1+ cells

B branch 1   **C** branch 2   **D** *Ascl2*

3d post Tam % of cells

66-70d post Tam % of cells

$P=0.001$

$P=0.001$

$P=10^{-8}$

3d post TAM

66-70d post TAM

Fig. 6

A

B log fold change

C Fraction of Cells Expressing CD74

D Expression CD74/cell

E Fatty Acid Metabolism, GSEA

F scATACseq *Cyp4a10*, Ec Cluster

G Trpv6 Expression enterocytes

H scATACseq *Trpv6*, Ec cluster

Enriched Pathways EC7, NWD1vAIN76A:
- antigen processing/presentation
- intestinal immune network, IgA production
- Ppar signaling
- cholesterol metabolism
- Fat digestion/absorption

A scATACseq clusters
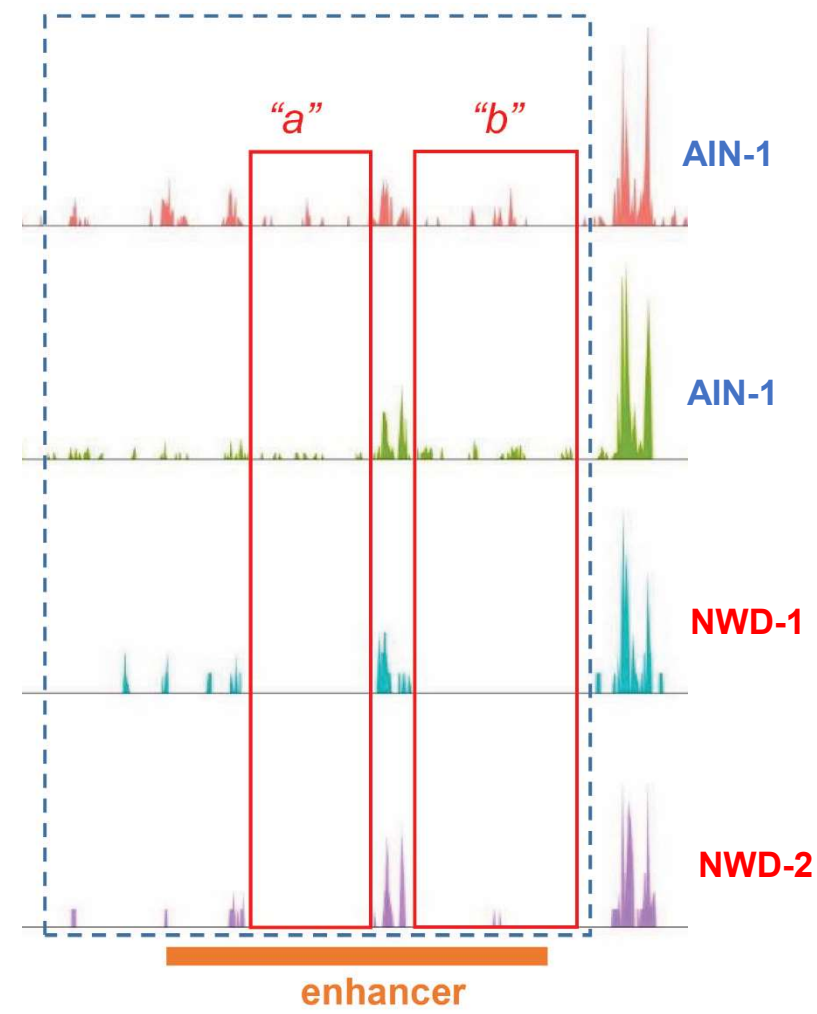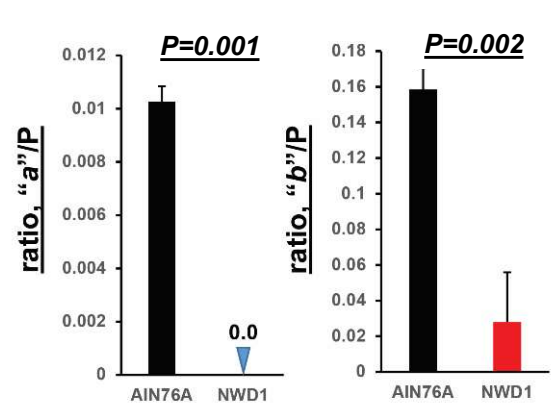
B % of cells in Stem 1

Fig. 7

C scATACseq for Ppargc1a in Stem 1
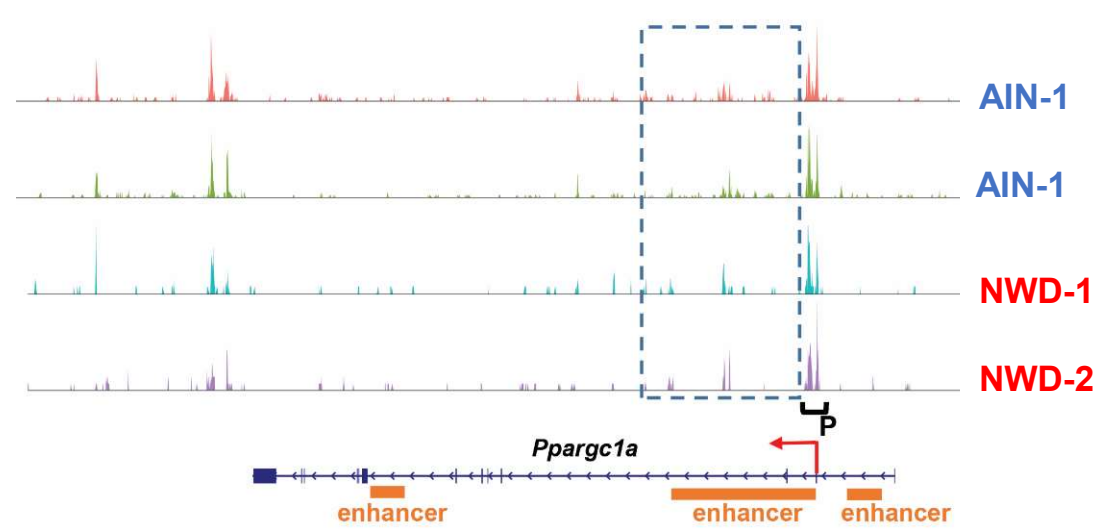
D region magnified

E quantification regions "a" and "b"

Supplemental Figures

Choi et al

Fig. S1

A

B
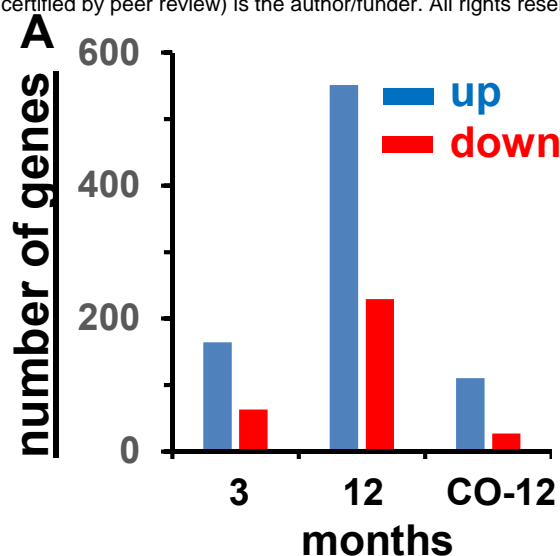
Pathway	3 months NWD1 v AIN			12 months NWD1 v AIN			12 month cross over back to AIN		
	NES	P	FDR	NES	P	FDR	NES	P	FDR
RIBOSOME	3.19	≤0.001	0.000	3.10	≤0.001	0.000	-3.13	≤0.001	0.000
DNA REPLICATION	2.41	≤0.001	0.000	2.26	≤0.001	0.000	-2.47	≤0.001	0.000
SPLICEOSOME	2.34	≤0.001	0.000	2.08	≤0.001	0.000	-2.34	≤0.001	0.000
RNA_POLYMERASE	2.19	≤0.001	0.000	1.88	≤0.001	0.001	-2.00	≤0.001	0.001
MISMATCH_REPAIR	2.13	≤0.001	0.000	2.14	≤0.001	0.000	-2.21	≤0.001	0.000
CELL_CYCLE	1.97	≤0.001	0.001	1.91	≤0.001	0.001	-2.08	≤0.001	0.000
NUCLEOTIDE EXCISION REPAIR	1.83	≤0.001	0.006	1.90	≤0.001	0.001	-1.78	0.003	0.009
PURINE METABOLISM	1.74	≤0.001	0.015	1.60	≤0.001	0.038	-1.54	0.002	0.079
OOCYTE MEIOSIS	1.73	0.002	0.016	1.42	0.030	0.160	-1.26	0.091	0.333
PYRIMIDINE METABOLISM	1.72	≤0.001	0.015	1.80	≤0.001	0.003	-1.67	≤0.001	0.026
BASE EXCISION REPAIR	1.68	0.004	0.020	2.09	≤0.001	0.000	-1.99	≤0.001	0.001
RNA DEGRADATION	1.68	≤0.001	0.019	1.63	0.006	0.031	-1.36	0.078	0.213
LINOLEIC ACID METABOLISM	1.66	0.004	0.020	1.46	0.062	0.153			
ARACHIDONIC ACID METABOLISM	1.59	0.015	0.038						
WNT SIGNALING PATHWAY	1.52	≤0.001	0.063						

Pathway	NES	P	FDR	NES	P	FDR	NES	P	FDR
ABC TRANSPORTERS	-2.34	≤0.001	≤0.001	-1.98	≤0.001	0.006	1.85	≤0.001	0.010
CITRATE CYCLE -TCA CYCLE	-2.28	≤0.001	≤0.001	-2.02	≤0.001	0.006	2.01	≤0.001	0.002
PEROXISOME	-2.20	≤0.001	≤0.001		≤0.001			≤0.001	≤0.001
OXIDATIVE PHOSPHORYLATION	-2.19	≤0.001	≤0.001	-2.37	≤0.001	≤0.001	2.11	≤0.001	0.001
PARKINSONS DISEASE	-2.14	≤0.001	≤0.001	-2.12	≤0.001	0.002	1.93	≤0.001	0.004
PPAR SIGNALING PATHWAY	-2.10	≤0.001	≤0.001		≤0.001				
FATTY ACID METABOLISM	-2.08	≤0.001	≤0.001		≤0.001				
GLYCOLYSIS GLUCONEOGENESIS	-2.07	≤0.001	≤0.001	-2.03	≤0.001	0.005			
RETINOL METABOLISM	-2.07	≤0.001	≤0.001		≤0.001				
ALZHEIMERS DISEASE	-2.02	≤0.001	≤0.001	-2.17	≤0.001	≤0.001	1.95	≤0.001	0.004
FRUCTOSE/MANNOSE METABOLISM	-1.98	≤0.001	≤0.001	-2.32	≤0.001	≤0.001	2.28	≤0.001	≤0.001
AMINO SUGAR/NUCLEOTIDE SUGAR METAB.	-1.92	0.002	0.002				1.72	0.010	0.023
GLYOXYLATE/DICARBOXYLATE METAB	-1.85	0.006	0.006						
SPHINGOLIPID METABOLISM	-1.84	≤0.001	0.006				1.89	0.005	0.008
BUTANOATE METABOLISM	-1.82	0.002	0.006						
OTHER GLYCAN DEGRADATION	-1.80	0.008	0.008	-1.53	0.058	0.133	2.12	≤0.001	≤0.001
PENTOSE PHOSPHATE PATHWAY	-1.78	0.004	0.010	-1.48	0.033	0.138			

Fig S2

TCA cycle

Oxphos

NWD1 3Mo vs AIN76A 3Mo

NES -2.3
P <0.001
FDR <0.001

NES -2.2
P <0.001
FDR <0.001

NWD1 12Mo vs AIN76A 12Mo

NES -2.0
P <0.001
FDR =0.006

NES -2.4
P <0.001
FDR <0.001

AIN76A crossover 12Mo vs NWD1 12Mo

NES +2.0
P <0.001
FDR =0.002

NES +2.1
P <0.001
FDR <0.001

Enrichment Profile ——
Gene Hits ——

Fig. S3

A

Cohort 1 ▬▬ Cohort 2 ▬▬

NWD1/AIN (3 months)

Oxphos genes (Kegg pathway),

	cohort 1	cohort 2
NWD1/AIN<1	58 (87%)	67 (100%)
Mean decrease	14%	11%

Cox6a2 *Cox7a1* *Sdhb* *Sdhd*

B

NWD1/AIN

▬▬ NWD1/AIN 12 months
▬▬ NWD1 cross over/AIN

Oxphos genes (Kegg pathway)

NWD1/AIN<1	65/67 (97%)
Mean decrease	10.0%
AIN Reversal	52 (82%)

Oxphos Kegg Pathway Genes

C

TCA cycle → complex I (17 subunits ↓) / complex II (3 subunits ↓) → Q → complex III (8 subunits ↓) → complex IV (14 subunits ↓) → ATPsynthase (17 subunits ↓)

electron flow

Fig S4

A. Trajectory, *Ppargc1a* inactivation
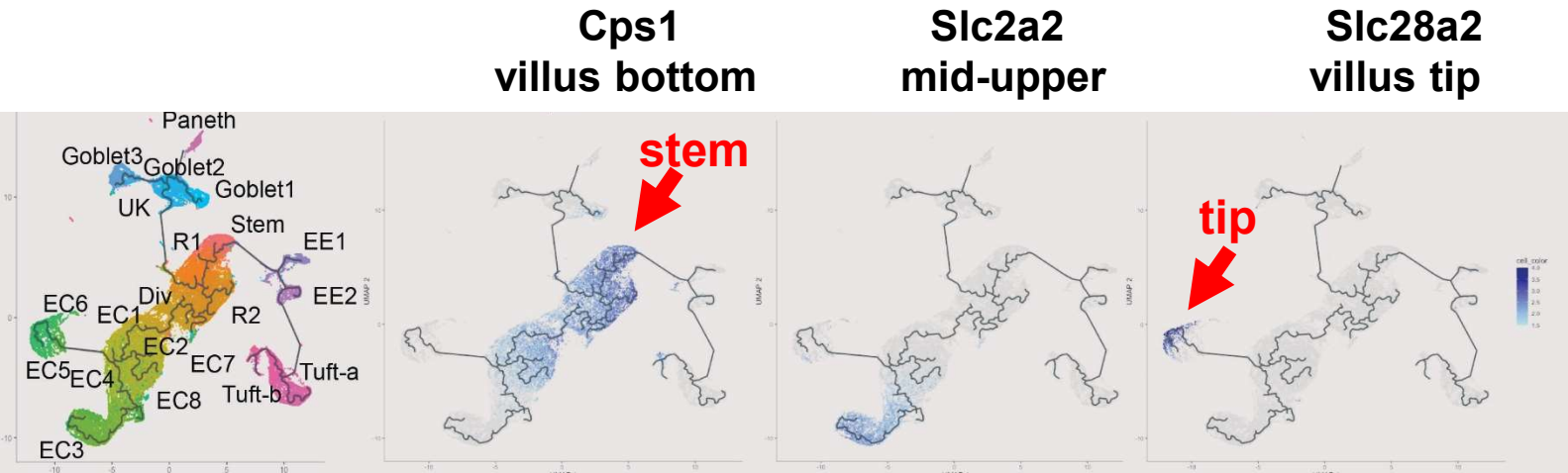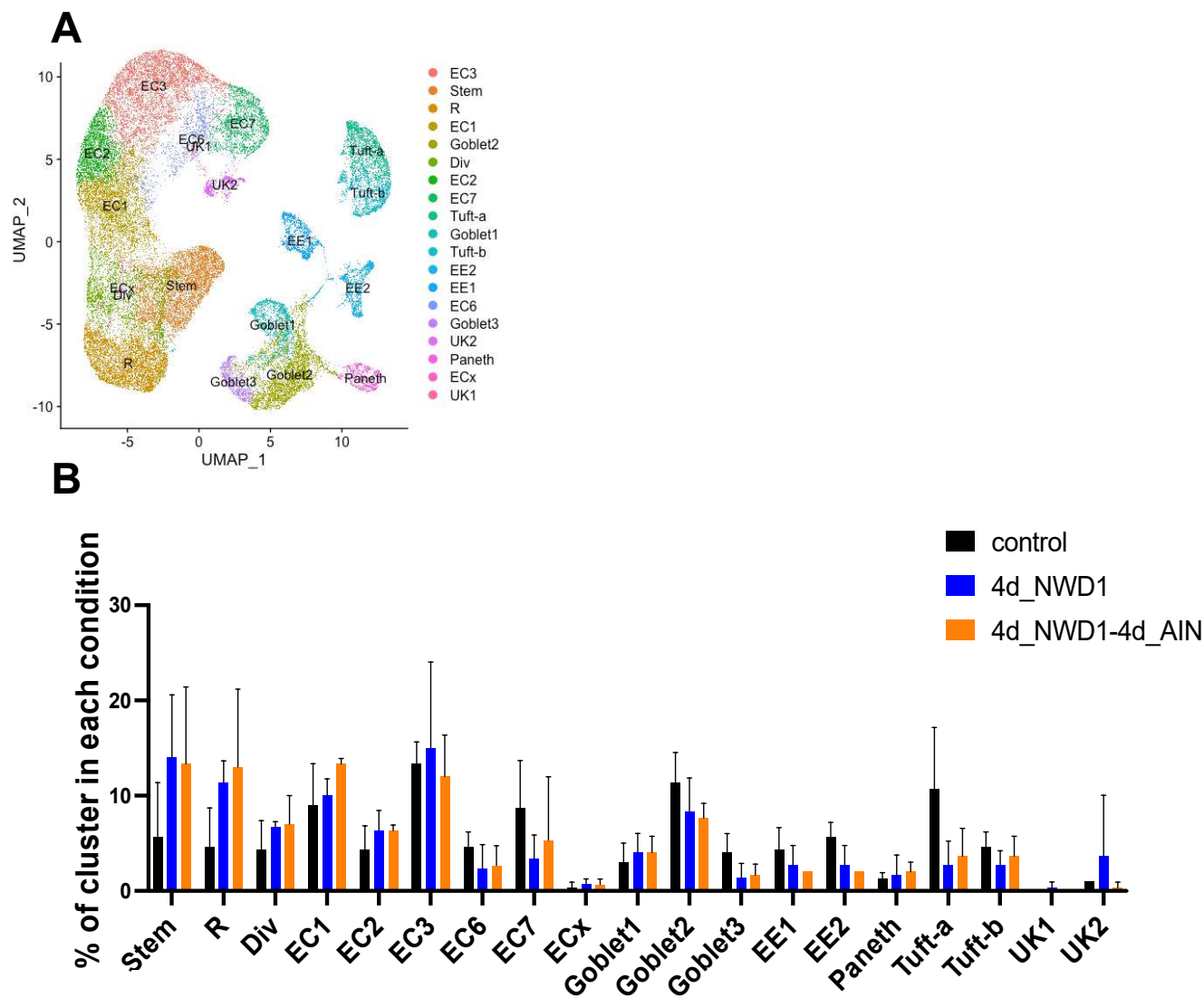

B. Trajectory, 4d dietary cross-over
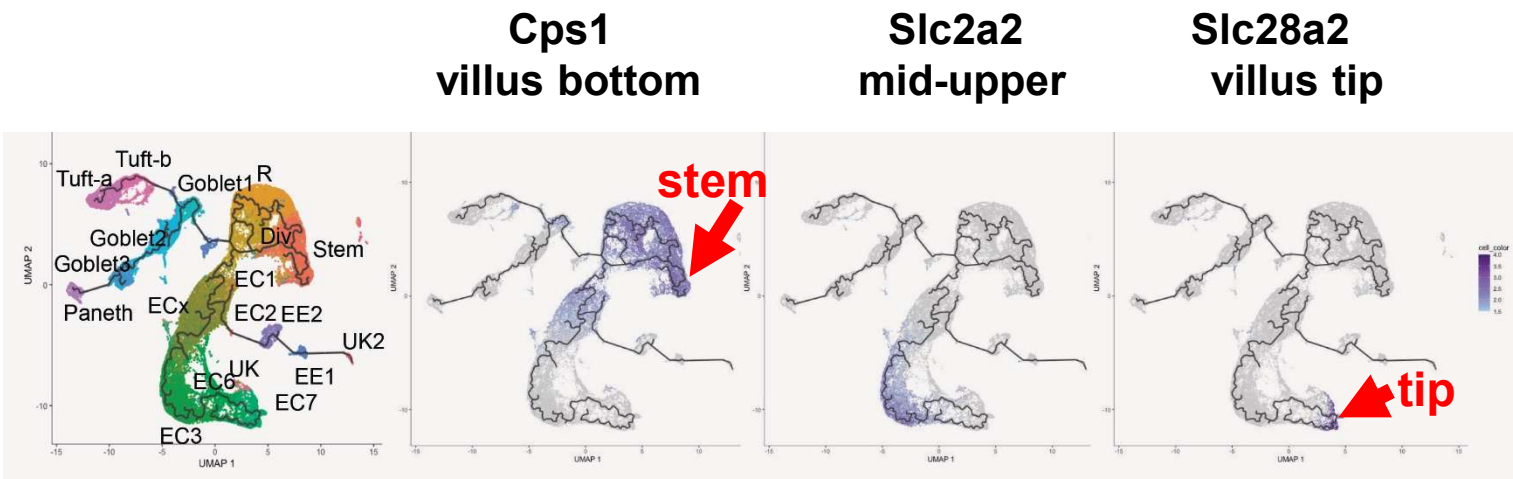
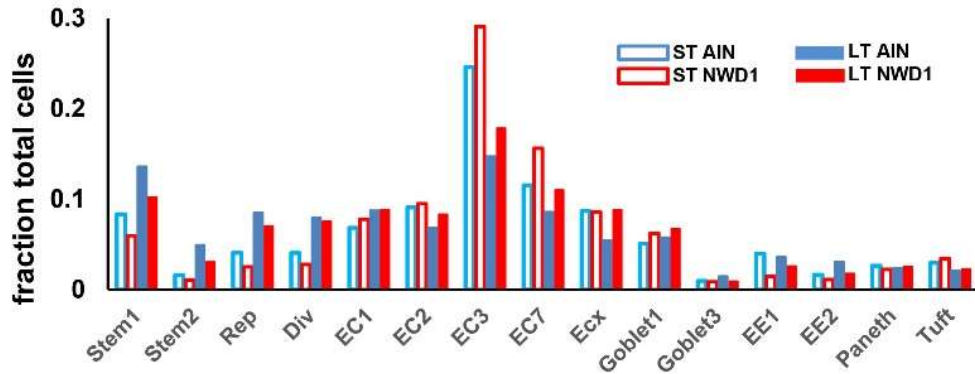## C. Trajectory, Bmi1+ cells
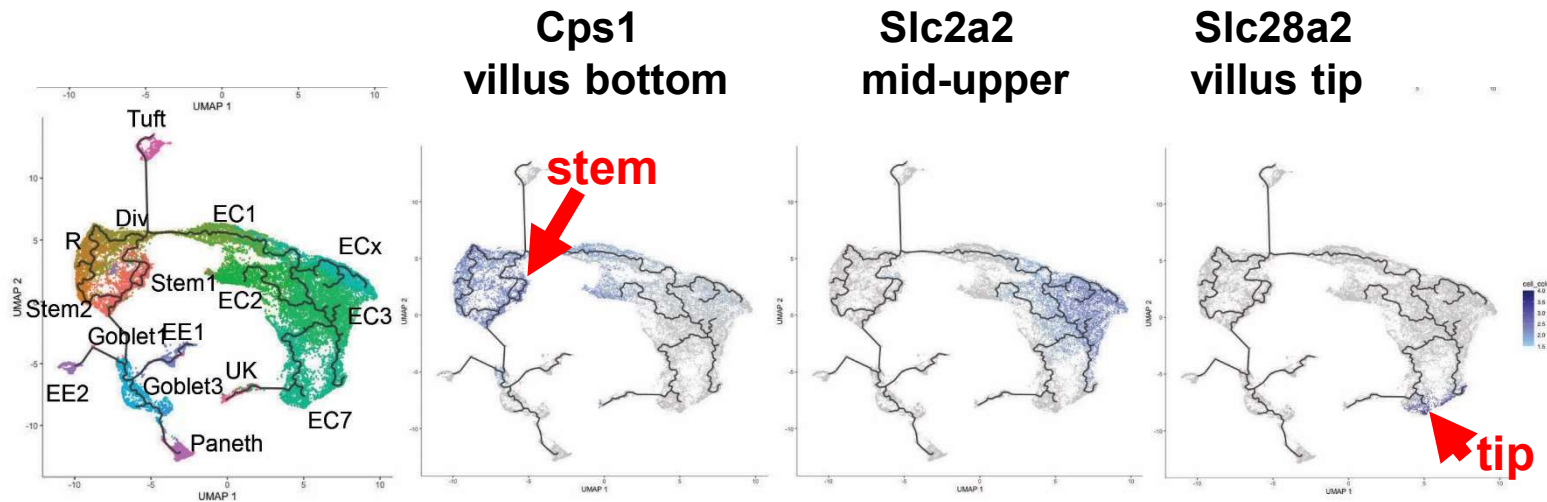
Cell Clusters, *Bmi1 Cells*

A

<u>scRNAseq libraries</u>

<u>diet (3mo)</u>	days <u>post-TAM</u>	
NWD1	3	
NWD1	3	<u>shorter term</u>
AIN76A	3	
AIN76A	3	
NWD1	66	
NWD1	70	<u>longer term</u>
AIN76A	66	
AIN76A	70	

B

C

D

Fig S7

A MHCII gene expression across all clusters

B CD3+ cells

C P=0.008

D P=0.015

E F/480+ cells

F P=0.01