

1 **Hyperactive WNT/CTNNB1 signaling induces a competing cell proliferation and**
2 **epidermal differentiation response in the mouse mammary epithelium**

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31 **ABSTRACT**

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33

34 In the past forty years, the WNT/CTNNB1 signaling pathway has emerged as a key player in mammary
35 gland development and homeostasis. While also evidently involved in breast cancer, much unclarity
36 continues to surround its precise role in mammary tumor formation and progression. This is largely
37 due to the fact that the specific and direct effects of hyperactive WNT/CTNNB1 signaling on the
38 mammary epithelium remain unknown. Here we use a primary mouse mammary organoid culture
39 system to close this fundamental knowledge gap. We show that hyperactive WNT/CTNNB1 signaling
40 induces competing cell proliferation and differentiation responses. While proliferation is dominant at
41 lower levels of WNT/CTNNB1 signaling activity, higher levels cause reprogramming towards an
42 epidermal cell fate. We show that this involves *de novo* activation of the epidermal differentiation
43 cluster (EDC) locus and we identify master regulatory transcription factors that likely control the
44 process. This is the first time that the molecular and cellular dose-response effects of WNT/CTNNB1
45 signaling in the mammary epithelium have been dissected in such detail. Our analyses reveal that the
46 mammary epithelium is exquisitely sensitive to small changes in WNT/CTNNB1 signaling and offer a
47 mechanistic explanation for the squamous differentiation that is observed in some WNT/CTNNB1
48 driven tumors.

49 INTRODUCTION

50

51 The mammary gland is a defining feature of all mammalian species. It is a dynamic tissue that
52 largely develops after birth, undergoing major changes in proliferation and differentiation during
53 puberty, pregnancy and, ultimately, lactation, when it supplies milk to the newborn offspring. While
54 largely driven by systemic hormones, local signaling cues are equally important to control cell division
55 and cell fate choices during tissue remodeling at each of these stages. Among these is the
56 WNT/CTNNB1 signal transduction pathway, which plays an essential role throughout development of
57 the tissue, from the earliest initiation of mammary placode formation (Chu et al., 2004; Veltmaat et al.,
58 2004) to adult mammary stem cell maintenance (Zeng and Nusse, 2010) and the promotion of side-
59 branching in early pregnancy (Brisken et al., 2000).

60 Hyperactivation of the WNT/CTNNB1 pathway promotes mammary tumor formation in mice
61 (Nusse and Varmus, 1982). Some, but not all of these tumors display features of squamous
62 differentiation (Miyoshi et al., 2002). How this characteristic is related to WNT/CTNNB1 activity
63 remains unknown due to the inherent latency of tumor development. The involvement of
64 WNT/CTNNB1 signaling in human breast cancer remains less clear (van Schie and van Amerongen,
65 2020). In general, the direct and immediate effects of elevated WNT/CTNNB1 signaling on the
66 mammary epithelium are poorly understood.

67 Here we take advantage of a primary organoid culture system that supports short-term growth
68 of the mammary epithelium in the absence of any exogenous growth factors, allowing us to modulate
69 activity of the WNT/CTNNB1 pathway and measure the consequences at a molecular and cellular level.
70 These experiments reveal that WNT/CTNNB1 signaling operates within a precise and narrow range to
71 offer a proliferative advantage to mammary epithelial cells, while higher levels rapidly induce
72 transdifferentiation of the mammary epithelium towards an epidermal cell fate.

73

74

75 RESULTS

76

77 **WNT/CTNNB1 signaling induces size and shape changes in the mammary epithelium**

78 The adult virgin mouse mammary gland is composed of a ductal epithelial network immersed
79 within a stromal fat pad that is mainly composed of adipocytes. The bilayered ductal epithelium
80 contains an outer layer of differentiated myoepithelial and less differentiated progenitor cells (jointly
81 referred to as basal cells) and an inner layer of mature luminal epithelial cells and luminal progenitors.

82 The two cell layers, which originate from a common embryonic progenitor, can be discriminated by the
83 expression of specific cytokeratins, with basal cells expressing KRT5 and KRT14 and luminal cells
84 expressing KRT8 (Figure 1A). Short-term primary organoid cultures of epithelial fragments grown in a
85 basement membrane matrix (Matrigel) and minimal media (Ewald et al., 2008) offer an accessible 3D
86 *in vitro* system for investigating how specific signaling cues affect growth of the mammary gland
87 parenchyma. This is especially relevant for studying the response to changes in WNT/CTNNB1 signaling,
88 since longer term cultures aimed at maintaining mammary epithelial stem cells require WNT/CTNNB1
89 pathway activation (Jardé et al., 2016; Sachs et al., 2018).

90 We followed established protocols (Nguyen-Ngoc et al., 2015), in which freshly digested
91 mammary epithelial fragments are embedded in growth-factor reduced matrigel and cultured in
92 minimal media devoid of WNT signals for 7 days (Figure 1B). In this controlled setup, we induced a
93 dose-dependent increase in WNT/CTNNB1 signaling using different concentrations of CHIR99021, a
94 specific small molecule GSK3 inhibitor that potently activates the WNT/CTNNB1 pathway downstream
95 of the WNT/FZD receptor complex (Figure 1C) (Naujok et al., 2014). While mammary organoids grown
96 under control conditions did not change morphology over the course of the experiment, organoids
97 cultured with increasing concentrations of CHIR99021 (1 μ M, 1.5 μ M and 3 μ M representing Wnt_{low},
98 Wnt_{med} and Wnt_{high} conditions) rapidly increased in size (Figure 1D, Supplementary Figure 1A). After 7
99 days, they were larger (Figure 1E, increase in Wnt_{low} vs control: 3.6-fold, P=4.09E-2; Wnt_{med} vs control:
100 7.3-fold, P=1.71E-8; Wnt_{high} vs control: 11.9-fold, P=2.51E-18), less circular (Figure 1F, control: mean
101 0.696, 95% CI 0.96-0.979; Wnt_{low}: mean 0.825, 95% CI 0.778-0.872; Wnt_{med}: mean 0.741, 95% CI 0.691-
102 0.791; Wnt_{high}: mean 0.878, 95% CI 0.818-0.937) and frequently contained one or more rounded
103 protrusions that appeared to emerge from the core structure (Figure 1D). The latter was more
104 prominent in organoids with intermediate levels of WNT/CTNNB1 signaling (Figure 1G), as also
105 reflected by their lower circularity. Considerable variation existed between independent experiments
106 (n=22 different mice) and individual organoids (Figure 1H, Supplementary Figure 1B, Supplementary
107 File 1).

108 To characterize the observed phenotypes in more detail, we performed immunofluorescence
109 staining on paraffin embedded organoids. We first probed the expression of two common basal and
110 luminal cell markers, K14 and K8, respectively. Vehicle-treated organoids were organized in two distinct
111 and well-organized cell compartments, with an outer layer of K14⁺ basal cells and an inner cell
112 population of K8⁺ luminal cells as previously described (Ewald et al., 2008). Most organoids also had a
113 defined lumen (Figure 1I). In contrast, organoids with hyperactive WNT/CTNNB1 signaling lost this
114 characteristic epithelial organization. After 7 days, CHIR99021-treated organoids showed separation of

115 the K8⁺ and K14⁺ cell populations, with the K8⁺ cells clustering on the outside, corresponding to the
116 regions where we observed the protrusions (Figure 1I, Supplementary Figure 2A). This is reminiscent
117 of tissue separation via differential adhesion (Foty and Steinberg, 2013). Especially at higher levels of
118 WNT/CTNNB1 signaling, individual nuclei also lost their original shape, size and orientation
119 (Supplementary Figure 2B).

120 Our combined observations so far suggest that the mouse mammary epithelium shows a
121 complex response to hyperactivation of the WNT/CTNNB1 pathway: The increase in size suggests a
122 proliferative response, while the morphological rearrangement of the basal and luminal compartments
123 suggests a change in cell identity. Of note, these changes occur within a narrow dose-response window,
124 since in our experimental system the difference between the Wnt_{high} (3 μ M CHIR99021) and Wnt_{low}
125 (1 μ M CHIR99021) conditions is only three-fold.

126 127 **Elevated levels of WNT/CTNNB1 signaling promote cell proliferation**

128 To obtain an unbiased view of the molecular events underlying the observed cellular changes,
129 we performed bulk RNA sequencing on three independent primary organoid cultures harvested at day
130 7, each containing all four experimental conditions (control, Wnt_{low}, Wnt_{med} and Wnt_{high}). Given the
131 variation we observed between experiments (Figure 1), we selected three experiments that covered
132 the full range of phenotypes (Supplementary Figure 1B, Supplementary Figure 3A,B), reasoning that
133 this should allow us to identify the most consistent transcriptomic changes. Differential gene
134 expression analysis revealed major changes between the four experimental treatments
135 (Supplementary File 1). Unsupervised hierarchical clustering suggested distinct thresholds for gene
136 activation and repression depending on the absolute levels of WNT/CTNNB1 signaling (Figure 2A).

137 We first confirmed that WNT/CTNNB1 signaling is indeed hyperactivated in CHIR99021 treated
138 organoids. To this end, we analyzed the response pattern of a curated set of 21 genes that have
139 previously been reported to be activated by WNT/CTNNB1 signaling in the mammary gland or in other
140 tissues (Boonekamp et al., 2021; Faflek et al., 2013; Szemes et al., 2018; Wang et al., 2015; Yu et al.,
141 2016). We made sure to include multiple bona-fide feedback targets, including *Axin2* and *Lgr5*
142 (Supplementary File 2). Out of these 21 genes, 14 were differentially expressed in one or more
143 conditions (Figure 2B). Most showed the expected dose-dependent induction, including *Axin2* and the
144 cell proliferation markers *Ccnd1* and *Myc*, although the absolute levels and dynamic range varied
145 between genes (Supplementary File 3). One notable exception was *Lgr5*, the expression of which was
146 consistently downregulated in all replicates (average 790-fold change in Wnt_{high} versus control, Figure
147 2C).

148 Compared to Wnt_{low} organoids, Wnt_{med} organoids were enriched for genes involved in cell cycle
149 progression (Figure 2D, Supplementary File 2, p-value = $1.43E-75$, FDR q-value = $4.61E-71$), most
150 notably the G2/M checkpoint (p-value = $1.07E-33$, FDR q-value = $5.35E-32$). Almost 90 differentially
151 expressed genes were classified as targets of the dimerization partner, RB-like, E2F and multi-vulval
152 class B (DREAM) complex, which regulates cell-cycle dependent gene expression (Sadasivam and
153 DeCaprio, 2013) (Supplementary File 2, p-value = $1.43e-75$, FDR q-value = $4.61e-71$). This includes
154 multiple E2F4 targets, such as *Top2a* and *Aurkb*, suggesting that their repression is relieved in response
155 to enhanced WNT/CTNNB1 signaling (Figure 2E). Of note, the comparison of Wnt_{low} and Wnt_{med}
156 expression profiles also revealed the induction of homologous recombination and Fanconi anemia
157 pathway genes, such as *Rad51*, *Brca1* and *Fancd2* (Figure 2F), which were recently shown to be induced
158 in WNT addicted cancers in a CTNNB1- and MYBL2/FOXO1 dependent manner (Kaur et al., 2021).
159 Indeed, both *Mybl2* and *FoxO1* were upregulated in Wnt_{med} organoids as well (Figure 2F), suggesting
160 that the same mechanism might operate in direct response to elevated levels of WNT/CTNNB1
161 signaling.

162 To confirm that the mammary epithelial cells indeed divide as a result of increased
163 WNT/CTNNB1 signaling, we performed lineage tracing in primary organoids derived from
164 *Axin2^{CreERT2};Rosa26^{Confetti}* mice (Van Amerongen et al., 2012; Snippert et al., 2010), in which Cre/lox
165 mediated recombination of a multi-color fluorescent reporter allele can be induced in cells that express
166 the WNT/CTNNB1 target gene *Axin2* (Figure 2G). In the combined presence of CHIR99021 (which
167 hyperactivates WNT/CTNNB1 signaling) and 4-hydroxytamoxifen (which induces Cre^{ERT2} recombinase
168 activity) clonal cell proliferation occurred in both basal and luminal regions, with large, flattened cells
169 accumulating in the center of the organoid (Figure 2H-I, Supplementary Figure 2C).

170 To validate our findings, we performed staining for the cell proliferation marker Ki67. This
171 showed that, as expected, very few cells were dividing in control treated organoids, with 2% of all
172 luminal cells and 6% of basal cells dividing (Supplementary File 3). Both dividing basal and luminal cells
173 could be detected in organoids with hyperactive WNT/CTNNB1 signaling, but they were largely absent
174 from the center (Figure 2J). Quantification of the number of dividing luminal and non-luminal cells
175 across the different conditions revealed the highest proportion of dividing cells in Wnt_{low} organoids
176 (mean 21% versus mean 2.5% in DMSO treated cells, $P=0.02$, Figure 2K), suggesting that the increase
177 in organoid size (highest in Wnt_{high} , Figure 1F) cannot solely be due to an increase in cell proliferation.
178 The overall proportion of luminal cells showed a slight but steady decrease with higher levels of
179 WNT/CTNNB1 signaling (mean 73% of all cells in DMSO versus mean 48% of all cells in Wnt_{high} , Figure
180 2L), but we did not detect statistically significant changes in the division of luminal and non-luminal

181 cells across the different treatment conditions (Figure 2M and Supplementary File 3). Thus, we
182 conclude that increased levels of WNT/CTNNB1 signaling promote cell division in both basal and
183 luminal mammary epithelial cells.

184

185 **Hyperactive WNT/CTNNB1 signaling induces squamous differentiation**

186 Returning to our transcriptomics analysis, we next asked if we could identify specific gene
187 signatures with distinct dose-dependent response patterns. To this end, we performed fuzzy c-means
188 clustering (Kumar and Futschik, 2007) on all genes that were differentially expressed in one or more
189 conditions. This allowed us to identify 12 clusters with co-regulated genes (Supplementary Figure 3C,D,
190 Supplementary File 2). Functional annotation and gene set enrichment analysis of individual clusters
191 revealed the dose-dependent and counterintuitive loss of a mammary stem cell signature (cluster 2,
192 Figure 3A, Supplementary File 2, LIM_MAMMARY_STEM_CELL_UP: p-value = 2.05e-14, FDR q-value =
193 5.02e-10) (Lim et al., 2010) and the loss of contractile features (cluster 10, Figure 3B, Supplementary
194 File 2, GOCC_CONTRACTILE_FIBER: p-value = 1.07e-26, FDR q-value = 1.73e-22). This included the loss
195 of myoepithelial cell markers *Acta2* and *Otxr* (Figure 3C, average fold reduction of 27.6 and 160
196 respectively in Wnt_{high} vs control, Supplementary File 3). The expression of basal keratins *Krt14* and
197 *Krt5* increased rather than decreased (Figure 3D, average fold increase of 12.1 and 9 respectively in
198 Wnt_{high} vs control, Supplementary File 3). Although this may partially be due to the loss of luminal cells,
199 this suggests that basal cells specifically lose their myoepithelial fate, but remain present as such, as
200 also supported by our immunostaining experiments (Figure 1I).

201 Among the gene clusters that predominantly increased in expression upon hyperactivation of
202 the WNT/CTNNB1 pathway, one stood out by showing hallmarks of squamous differentiation, which
203 included the gain of a squamous breast tumor signature (cluster 3, Figure 3E, Supplementary File 2,
204 HOLLERN_SQUAMOUS_BREAST_TUMOR: p-value = 4.8e-31, FDR q-value = 7.75e-27). Closer inspection
205 revealed upregulation of multiple genes associated with epidermal development, keratinization and
206 cornification (cluster 3, Supplementary File 2, GOBP_CORNIFICATION: p-value = 4.79e-13; FDR q-value
207 = 7.03e-10, GOBP_KERATINIZATION: p-value = 5.43e-13; FDR q-value = 7.63e-10,
208 GOBP_EPIDERMIS_DEVELOPMENT: p-value = 2.33e-12; FDR q-value = 2.42e-09). Indeed, H&E stained
209 organoids sections revealed a dense core of eosinophilic material in the center of Wnt_{high} but not
210 Wnt_{low} organoids (Figure 3F), resembling keratin pearls that are characteristic of squamous
211 differentiation *in vivo* (Supplementary Figure 2D). While squamification was most apparent in the
212 Wnt_{high} condition, a squamous gene expression signature was also already present in Wnt_{low} organoids
213 (Supplementary File 2, Figure 3G). This suggests that squamous differentiation and proliferation of the

214 mammary epithelium are induced in parallel, with the squamous phenotype becoming more dominant
215 at higher levels of WNT/CTNNB1 signaling.

216

217 **Reprogramming of the mouse mammary epithelium towards an epidermal cell fate**

218 The mammary gland develops as a skin appendage. Like the hair follicle, it starts as a local
219 thickening of the surface ectoderm. WNT signaling is required for the initiation of both mammary and
220 hair follicle placode formation (Andl et al., 2002; Chu et al., 2004) and hyperactivation of WNT/CTNNB1
221 signaling in the epidermis, via the expression of a dominant active form of CTNNB1 under the control
222 of a *Krt14* promoter, is sufficient to drive *de novo* hair follicle formation and gives rise to hair tumors,
223 or pilomatricomas, in older mice (Gat et al., 1998). At the same time, WNT/CTNNB1 signaling also
224 controls growth and maintenance of non-hairy and interfollicular epidermis (Choi et al., 2013; Lim et
225 al., 2013). We therefore asked if the squamous differentiation we observed reflected reprogramming
226 of the mammary epithelium towards either of these cell fates.

227 Expression of the hair matrix markers *Lef1*, *Shh* and *Ptch1* (Gat et al., 1998) did not change in
228 response to CHIR99021 treatment, but we did detect increased expression of genes encoding
229 epidermal keratins (*Krt1* and *Krt10*) and skin barrier proteins (*Flg*) (Figure 3H, Supplementary File 3).
230 Immunofluorescence staining confirmed the presence of KRT10-positive cells immediately adjacent to
231 KRT5-positive basal cells as well as the presence of Loricrin (LOR) positive cells in the center of both
232 WNT_{med} and WNT_{high} organoids (Figure 3I). This pattern of expression is identical to that observed in
233 stratified epithelia, where KRT10 is expressed in the first suprabasal (or spinous) layer and where LOR
234 expression switches on in the upper spinous and lower granular layer. These differentiating cells no
235 longer divide, thereby also explaining the lack of proliferation in this area (compare Figure 2J and 3I,
236 Supplementary Figure 2D). Together, these findings suggest that supraphysiological levels of
237 WNT/CTNNB1 signaling induce transdifferentiation of the mammary epithelium towards an epidermal
238 cell fate.

239 To find further support for this hypothesis, we took advantage of the existence of scRNAseq
240 gene expression signatures that distinguish different parts of the epidermis and anagen hair follicle
241 (Joost et al., 2020). We investigated the (changes in) expression of the top 20 genes (Supplementary
242 File 2) that characterize 33 distinct subpopulations in either the permanent epidermis (9/33 clusters)
243 or the anagen hair follicle (24/33 clusters). CHIR99021 treated mammary organoids specifically induce
244 markers that characterize the (cycling) basal and suprabasal interfollicular epidermis and the
245 suprabasal upper hair follicle, but not of the sebaceous gland, outer bulge or hair germ (Supplementary
246 Figure 4A-I). CHIR99021 treated organoids also expressed some markers that characterize different

247 parts of the cycling anagen hair follicle (Supplementary Figure 4J-AG). These were mostly associated
248 with actively dividing populations, however (Supplementary Figure 4P-S). Their expression (e.g. *Top2a*,
249 *Ccnb1*, *Cenpf*) therefore likely reflects the increased cell division we observe, rather than a distinct cell
250 fate.

251 Closer inspection of the observed gene expression changes revealed that in addition to *Krt1* and
252 *Krt10*, the highest increase in keratin gene expression was observed in a select number of keratin genes
253 (*Krt6a*, *Krt6b*, *Krt16*, *Krt17*, Supplementary File 3) that are typically induced in interfollicular epidermis
254 that is stressed or wounded (Zhang et al., 2019). No such increase was observed for keratin genes that
255 characterize other stratified epithelia, such as the palmoplantar epidermis (*Krt9*) (Schweizer et al.,
256 1989) or the oral and esophageal epithelium (*Krt4*, *Krt13*) (van Muijen et al., 1986; Trisno et al., 2018).

257 While analyzing our RNAseq data, we realized that many of the genes associated with the
258 epidermal, keratinization and cornification gene signatures (i.e. cluster 3) were located in the same
259 region, namely the epidermal differentiation complex (EDC) locus on mouse chromosome 3q (Figure
260 4A). Spanning more than 3Mb, the EDC harbors ~60 genes that play a critical role in terminal
261 differentiation of the epidermis. It contains 4 distinct gene families, with the *S100* genes found at the
262 5' and 3' border and the small proline-rich (*Sprr*) genes, late cornified envelope (*Lce*) genes and *Flg*-like
263 genes (e.g. *Flg*, *Rptn* and *Tchh*) located in between. While the most proximal and distal *S100* genes
264 (*S100a1*, *S100a13*, *S100a11* and *S100a10*) were expressed in control treated organoids and remained
265 expressed at comparable levels irrespective of CHIR99021 treatment (Figure 4A,B), expression of the
266 intervening genes was low to undetectable in DMSO treated organoids. Upon hyperactivation of the
267 WNT/CTNNB1 pathway, however, 55 genes belonging to different families (e.g. *Sprr1a*, *Lce1b* and *Rptn*)
268 dramatically increased in expression (Figure 4A,C). This suggests that the entire EDC becomes
269 activated, as would be expected for epidermal keratinocytes. Taking everything together, we conclude
270 that hyperactive WNT/CTNNB1 signaling in primary mammary organoids is sufficient to reprogram cells
271 to an epidermal state, after which the reprogrammed cells undergo the normal differentiation program
272 of basal keratinocytes.

273

274 **WNT/CTNNB1 signaling induces master regulators of keratinocyte differentiation**

275 So how does WNT/CTNNB1 signaling transform mammary epithelial cells towards an epidermal
276 fate? We reasoned that this massive change in gene expression likely requires the activity of one or
277 more master regulators of epidermal differentiation. Using gene set enrichment analysis for the 227
278 differentially expressed genes from cluster 3, which gave rise to the squamous gene signature
279 (Supplementary File 2, Supplementary Figure 3), as well as the 55 differentially expressed EDC genes

280 (Figure 4A), we generated a shortlist of candidate transcription factors (Supplementary File 3). Of the
281 top-ranked candidates, 6 were shared between the two queried gene sets (Figure 4D). All 6 were dose-
282 dependently induced in CHIR99021 treated mammary organoids (Figure 4E). We selected two of these
283 genes (*Ovol1* and *Grhl3*, typically expressed in basal and suprabasal keratinocytes, respectively),
284 together with *Klf4* for experimental follow-up based on their known involvement in skin barrier
285 formation (Segre et al., 1999; Teng et al., 2007; Ting et al., 2005) and their proposed role in EDC locus
286 regulation (Klein et al., 2017; Nascimento et al., 2011). Using published ChIPseq data, we detected
287 multiple common TCF/LEF binding sites close to the transcriptional start site of all three genes (Figure
288 4F). We therefore speculate that WNT/CTNNB1 signaling directly induces a squamous differentiation
289 program by binding to regulatory elements that control the expression of these master regulatory
290 transcription factors.

291 To validate that WNT/CTNNB1 signaling indeed induces the expression of *Ovol1*, *Grhl3* and *Klf4*
292 prior to activating EDC locus genes, we stimulated the WNT-responsive BC44 mouse mammary
293 epithelial cell line (Deugnier et al., 1999) with either 3 μ M CHIR99021 or 50 ng/ml purified WNT3A
294 protein for 4 or 24 hours. Next, we determined the expression of the three putative master regulators
295 (*Ovol1*, *Klf4* and *Grhl3*) as well as three EDC locus genes (*Sprr1b*, *Tchh* and *Rptn*) by quantitative RT-
296 PCR. After 4 hours, both CHIR99021 and purified WNT3A induced the expression of endogenous *Ovol1*,
297 *Klf4* and *Grhl3* but not endogenous *Sprr1b*, *Rptn* and *Tchh* above baseline (Figure 4G, Supplementary
298 File 3). After 24 hours, the EDC locus genes were also induced, with endogenous *Rptn* showing the
299 highest fold change (47-fold increase in WNT3A treated cells versus control, Figure 4G, Supplementary
300 File 3). Of note, transient overexpression of GRHL3, but not OVOL1 or KLF4, was sufficient to induce
301 endogenous *Rptn* but not its neighboring gene, *Tchh* (Figure 4H, Supplementary File 3). This is in line
302 with the fact that *Rptn* was previously suggested to be a conserved target gene for GRHL transcription
303 factors in both mouse and human (Mathiyalagan et al., 2019).

304

305

306 DISCUSSION

307

308 Using a short-term, 3D primary organoid culture system, we have dissected the early response
309 of the mouse mammary epithelium to elevated levels of WNT/CTNNB1 signaling (Figure 1). We show
310 that hyperactive WNT/CTNNB1 signaling is sufficient to induce proliferation of both basal and luminal
311 cells (Figure 2). At the same time, increased levels of WNT/CTNNB1 signaling induce squamous
312 differentiation (Figure 3). These two activities compete with each other, resulting in specific changes

313 in organoid size, shape and cellular composition that depend on the absolute levels of WNT/CTNNB1
314 signaling. At lower levels, proliferation has the upper hand, while at higher levels differentiation
315 becomes dominant (Figure 4I). Of note, these events become apparent within 3 days and occur within
316 a narrow dose-response window (Supplementary Figure 1).

317 The squamous differentiation we observe in our primary organoids resembles the phenotype
318 that was previously reported for a 3D culture system aimed at allowing long-term passaging of mouse
319 mammary organoids. Here, organoids are grown in the presence of RSPO1 (Jardé et al., 2016),
320 suggesting that slight amplification of the endogenous WNT/CTNNB1 signaling activity is already
321 sufficient to transform the mouse mammary epithelium into epidermis. We hypothesize that the
322 consistent and major reduction in *Lgr5* expression levels that we observe in response to WNT/CTNNB1
323 hyperactivation (Figure 2C) reflects an attempt of the mammary epithelium to bring WNT/CTNNB1
324 signaling levels back down into to the physiological range. Given that *Lgr5* is typically considered to be
325 a positive WNT/CTNNB1 feedback regulator (Barker et al., 2007), this underscores the dynamic
326 adaptation of such feedback loops.

327 Our data suggest that the squamous differentiation signature reflects reprogramming of the
328 mammary epithelium towards an epidermal cell fate. This transdifferentiation event involves
329 activation of the EDC locus (Figure 4), which normally only occurs in differentiating keratinocytes and
330 which typically involves physical relocation of the EDC locus from the nuclear periphery to the nuclear
331 interior. This requires major changes in chromatin organization and (super) enhancer activity and is
332 thought to be regulated by the *Trp63*-mediated induction of the SWI/SNF chromatin remodeling factor
333 *Smarca4* (previously called *Brg1*) (Mardaryev et al., 2014), while also possibly involving GRHL3 (Klein
334 et al., 2017; Poterlowicz et al., 2017). Recent work suggests that reduced tension of the nuclear lamina
335 in suprabasal keratinocytes, resulting from the loss of ITGB1 attachment to the extracellular matrix,
336 can also directly induce physical relocation and transcriptional activation of the EDC locus (Carley et
337 al., 2021). Interestingly, we also observe changes in nuclear shape and size in response to hyperactive
338 WNT/CTNNB1 signaling (Supplementary Figure 2). The precise nature of this event remains unknown,
339 but it could well be either a cause or consequence of reprogramming towards and epidermal cell fate.

340 Furthermore, our data show that WNT/CTNNB1 signaling in and by itself is sufficient to
341 jumpstart a complex gene regulatory network that involves multiple master regulators of epidermal
342 differentiation (Figure 4D-H). Together, these transcription factors are well known to control epidermal
343 differentiation and other ectodermal developmental processes (Figure 4I) (Dai et al., 1998; Ferretti et
344 al., 2011; Kimura-Yoshida et al., 2015; Koster et al., 2004; Nair et al., 2006; Romano et al., 2012). Both
345 *Irf6* and *Znf750* are known TRP63 target genes. They, in turn, are thought to induce the expression of

346 *Klf4* and *Grhl3* as well as terminal differentiation genes (Moretti et al., 2010; Oberbeck et al., 2019; Sen
347 et al., 2012). *Ovol1* and *Trp63* have previously been shown to be directly activated by WNT/CTNNB1
348 signaling (Ferretti et al., 2011; Li et al., 2002). *Grhl3* has also previously been suggested to be a direct
349 WNT target gene in osteoblasts (Salazar et al., 2016). Our data suggest that a similar gene regulatory
350 network can be induced in mammary epithelial cells.

351 Of course, many questions remain. First and foremost, what ultimately shifts the balance from
352 proliferation to differentiation remains to be determined (Figure 4J). Mechanistically, the proliferation
353 response is characterized by an expression signature that is enriched for genes involved in the G2/M
354 checkpoint and DNA repair (Figure 2). On the one hand, this could indicate that WNT/CTNNB1 signaling
355 operates to enhance DNA repair, as recently suggested (Kaur et al., 2021). On the other hand, it is
356 tempting to speculate that this signature actually reflects replication stress. This would fit with an
357 earlier observation that increased WNT signaling induces a DNA damage response in primary human
358 mammary epithelial cells (Ayyanan et al., 2006) and could explain why higher levels of WNT/CTNNB1
359 signaling do not continue to offer a proliferative advantage. This would also fit with the induction of
360 stress keratins (Supplementary File 3). In fact, it might be directly connected to the squamous
361 differentiation phenotype, as keratinocytes are known to undergo differentiation in response to DNA
362 damage and replication stress (Freije et al., 2014; Molinuevo et al., 2020).

363 One caveat of the current study is that we have not yet resolved the earliest temporal changes
364 in gene expression nor the precise nature of CTNNB1-dependent DNA binding events. Second, it will
365 be interesting to see if a similar response can be detected *in vivo* and, related to this, if basal and
366 luminal cells respond differently. In our experimental setup we cannot clearly discriminate the behavior
367 of basal and luminal cells. While both seem to proliferate in response to hyperactive WNT/CTNNB1
368 signaling (Figure 2), it is not yet clear if both also undergo squamous differentiation. While basal cells
369 may seem more likely to transdifferentiate, the mammary epithelium develops from a common
370 embryonic progenitor (Spike et al., 2012; Wansbury et al., 2011) and both basal and luminal cells have
371 great inherent plasticity (Van Keymeulen et al., 2015; Koren et al., 2015).

372 Finally, a logical next question is in how far our findings are relevant for the human mammary
373 epithelium and the development or treatment of breast cancer. In mice, only low to intermediate levels
374 of WNT/CTNNB1 signaling are able to drive mammary tumor formation, with higher levels invariably
375 leading to squamous differentiation and metaplastic tumors (Edwards et al., 1992; Miyoshi et al., 2002;
376 Monteiro et al., 2014; Tsukamoto et al., 1988). In humans, activating genetic mutations resulting in
377 high levels of WNT/CTNNB1 signaling are typically only found in a subset of metaplastic breast
378 carcinomas (Hayes et al., 2008; Ng et al., 2017). Both growth promoting and inhibitory effects of

379 paracrine WNT signaling, including a squamous differentiation response in the latter, have been
380 reported in patient derived xenografts (Green et al., 2013). While squamous differentiation has been
381 reported to occur in human breast cancer (Tsuda et al., 1997), it is not considered to be common. At
382 the same time, mouse squamous tumors and human basal tumors share some gene expression
383 features (Hollern et al., 2018). If and how this correlates to active WNT/CTNNB1 signaling remains
384 unknown. Determining WNT-responsive gene expression signatures for the healthy and cancerous
385 human breast epithelium will be a critical first step to shed more light on this matter. Our data would
386 predict that here too, subtle changes in WNT/CTNNB1 signaling will affect cell proliferation and
387 differentiation in parallel.

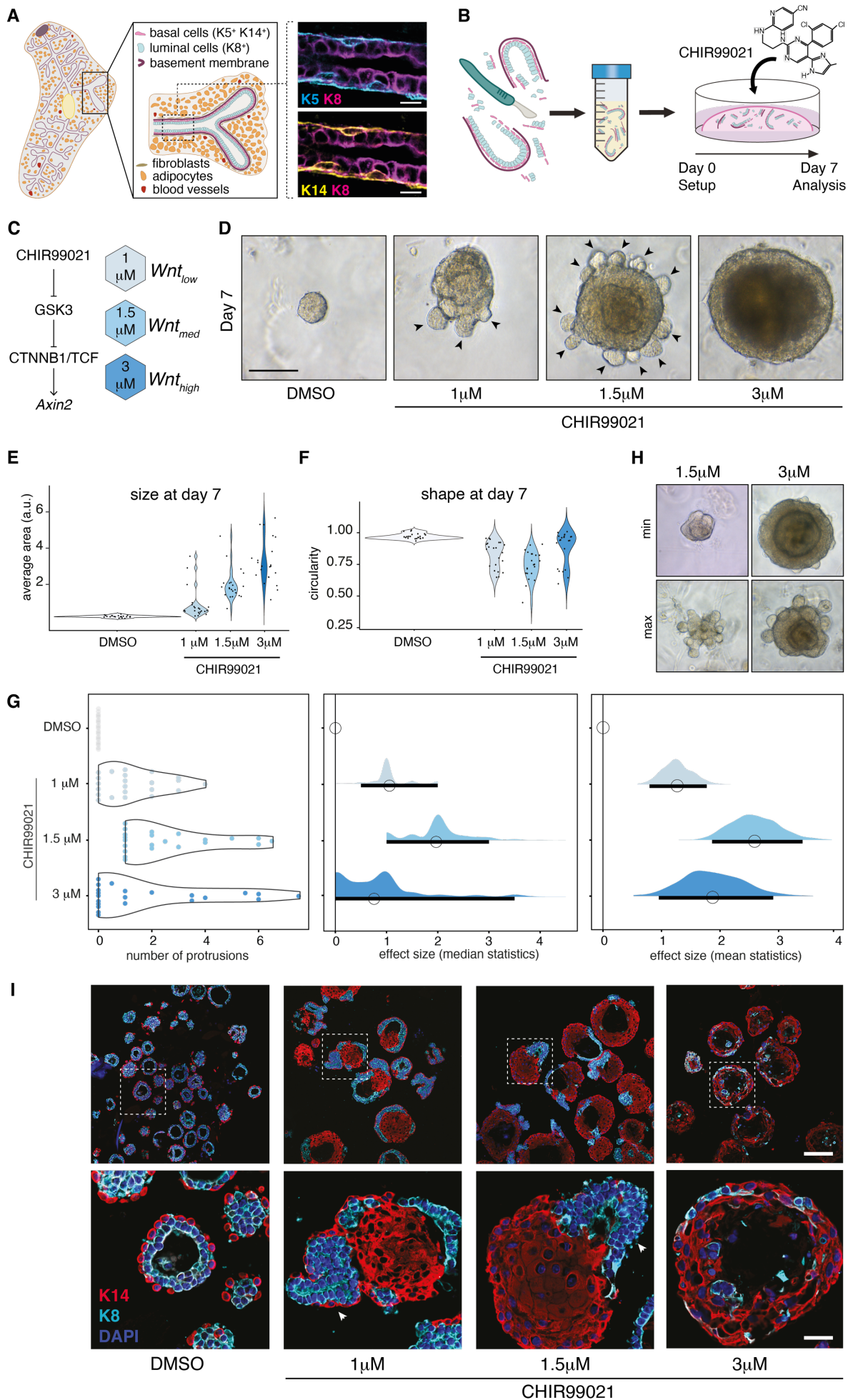
388 In conclusion, our findings highlight the sensitivity of the mammary epithelium to small changes
389 in WNT/CTNNB1 signaling and offer a mechanistic explanation for the selection of ‘just right’ levels of
390 WNT/CTNNB1 signaling in mammary tumor formation (Gaspar et al., 2009; van Schie and van
391 Amerongen, 2020). Accordingly, we hypothesize that human breast tumors will have a Wnt_{low} signature
392 that promotes proliferation. In contrast, cells with a Wnt_{high} signature, which induces reprogramming
393 towards an epidermal phenotype and results in squamous differentiation, are likely to be counter
394 selected at an early stage of tumor development.

395

396

397 **FIGURES AND FIGURE LEGENDS**

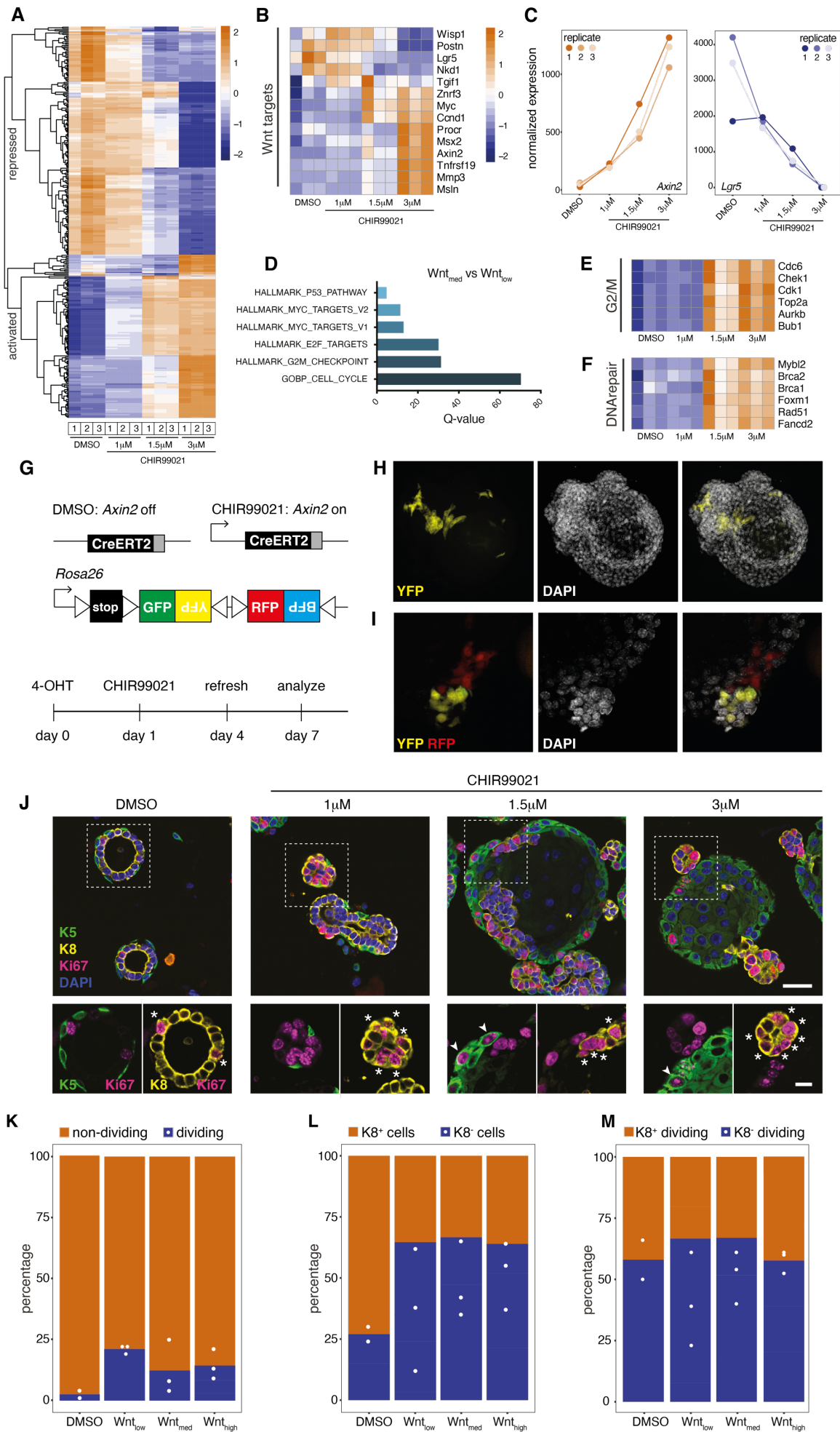
398



399 **Figure 1 Hyperactivation of the WNT/CTNNB1 pathway induces shape and size changes in the mouse mammary**
400 **epithelium**

401 A) Cartoon illustrating the cellular composition of the mouse mammary gland and close up of an immunofluorescent
402 staining of the ductal epithelium, depicting the basal (KRT5+ or KRT14+) and luminal (KRT8+) cell layers. Scalebar = 10 μm .
403 B) Schematic illustrating the experimental set up of the 3D primary organoid culture system in which epithelial fragments
404 are embedded in growth-factor reduced matrigel and grown in minimal media for 7 days. C) Addition of the small molecule
405 GSK3 inhibitor CHIR99021 allows dose-dependent hyperactivation of WNT/CTNNB1 signaling, resulting in WNT_{low} (1 μM),
406 WNT_{med} (1.5 μM) and WNT_{high} (3 μM) conditions. D) Representative brightfield microscopy image illustrating the organoid
407 phenotype after 7 days of control (DMSO) or CHIR99021 treatment. Arrowheads point at protrusions. Scalebar = 100 μm .
408 E-F) Violin plots depicting E) the increase in size and F) the change in shape as measured on brightfield microscopy images
409 taken on day 7. Data from n=22 independent organoid cultures are plotted. G) Plots of differences showing (left) the
410 number of protrusions observed (each data point represents the median number of protrusions for one of n=22
411 independent organoid cultures) and the calculated effect sizes using (middle) median and (right) mean statistics. H)
412 Brightfield microscopy image illustrating the variation in the phenotypes observed in independent organoid cultures. Min
413 = minimal protrusion formation. Max = maximal protrusion formation. I) Confocal microscopy image of an
414 immunofluorescent staining of formalin fixed, paraffin embedded sections of agarose-mounted organoid cultures on day
415 7. K14 = KRT14 (basal marker), K8 = KRT8 (luminal marker), DAPI = nuclei. Arrowheads point at protrusions. Scalebar = 100
416 μm (overview) and 25 μm (inserts). Source data and statistics provided in Supplementary File 3.

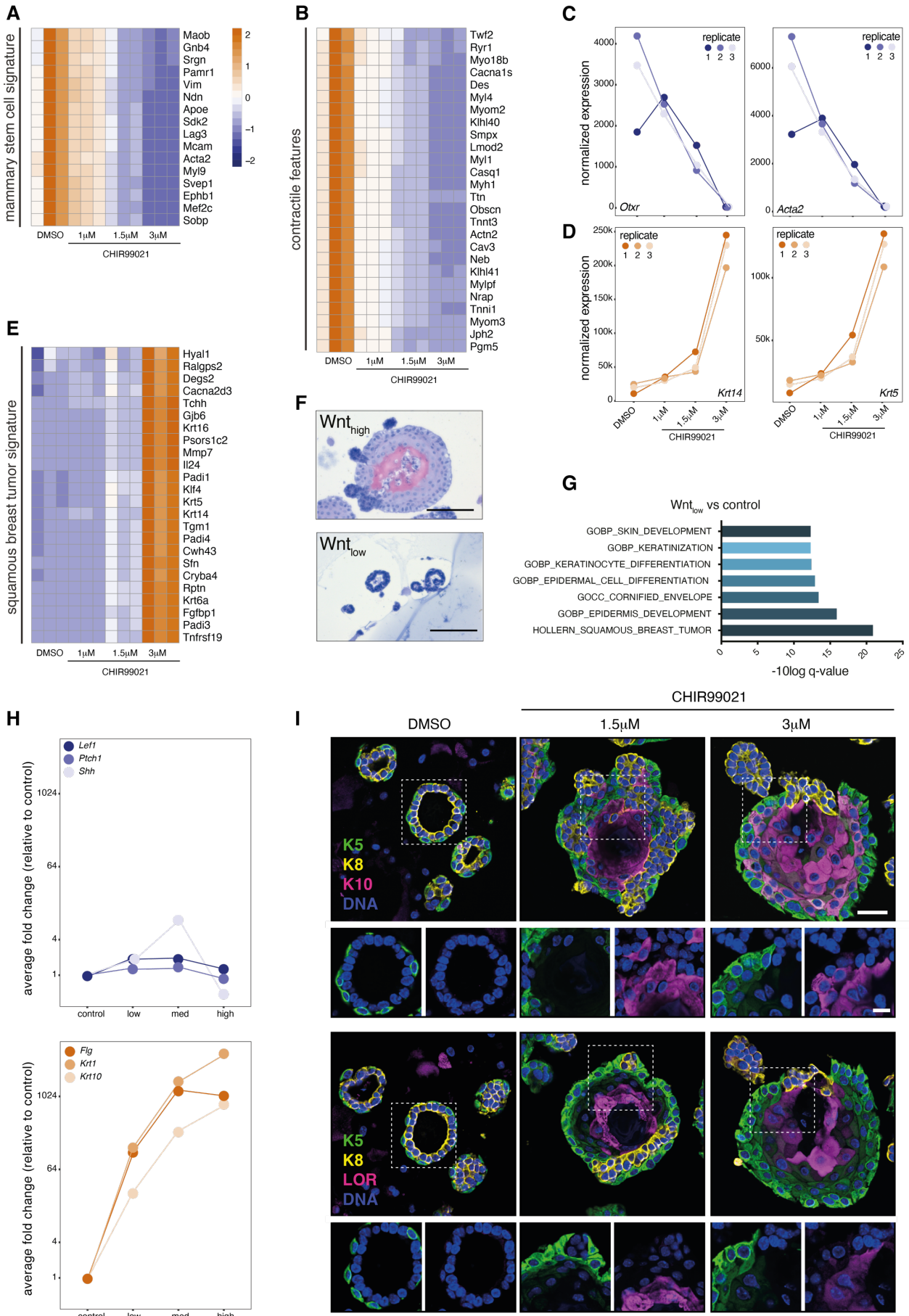
417



418 **Figure 2 Low levels of WNT/CTNNB1 signaling induce proliferation of basal and luminal cells**

419 A) Heatmap (unsupervised clustering, log₂-transformed normalized expression values, Z-score) showing distinct thresholds
420 for gene expression changes at different levels of WNT/CTNNB1 signaling activity. For this and all following heatmaps,
421 RNAseq results for n=3 independent organoid cultures are depicted for all treatment conditions. B) Heatmap (unsupervised
422 clustering, Z-score) of WNT/CTNNB1 target genes that are differentially expressed in one or more conditions. C) Graph
423 showing normalized expression values of the three RNAseq replicates for two of the WNT/CTNNB1 target genes from B):
424 *Axin2* (left) and *Lgr5* (right). D) Bar plot depicting the results of a gene set enrichment analysis for genes that are
425 differentially expressed in WNT_{med} (1.5 μM CHIR99021) versus WNT_{low} (1 μM CHIR99021) organoids. E-F) Heatmaps
426 (unsupervised clustering, Z-score) showing a selection of differentially expressed genes involved in E) the G2/M checkpoint
427 and F) DNA repair. G) Schematic illustrating the lineage tracing setup for the experiments depicted in H-I. A tamoxifen-
428 inducible CreERT2 recombinase is expressed in *Axin2*-positive cells, allowing recombination of a *Rosa26-Confetti* multicolor
429 reporter allele in cells with active WNT/CTNNB1 signaling (i.e. only in the presence of CHIR99021). H-I) Confocal microscopy
430 images showing clonal outgrowth of WNT/CTNNB1-responsive cells using the setup depicted in G. J) Confocal microscopy
431 image of an immunofluorescent staining of formalin fixed, paraffin embedded sections of agarose-mounted organoid
432 cultures on day 7. K5 = KRT5 (basal marker), K8 = KRT8 (luminal marker), KI67 = cell proliferation marker, DAPI = nuclei.
433 Asterisks point to dividing luminal cells. Arrowheads point to dividing basal cells. Scalebar = 25 μm (overview) and 10 μm
434 (inserts). K-M) Stacked bar graphs showing quantification of n=2 (DMSO) and n=3 (CHIR) independent experiments similar
435 to the one depicted in J. Total cell numbers counted per experiment (regions of interest (ROI) based on DAPI signal): DMSO:
436 655 and 1957; WNT_{low}: 529, 9473 and 1261; WNT_{med}: 6294, 240 and 1675; WNT_{high}: 1942, 478 and 398. Source data and
437 statistics provided in Supplementary File 3.

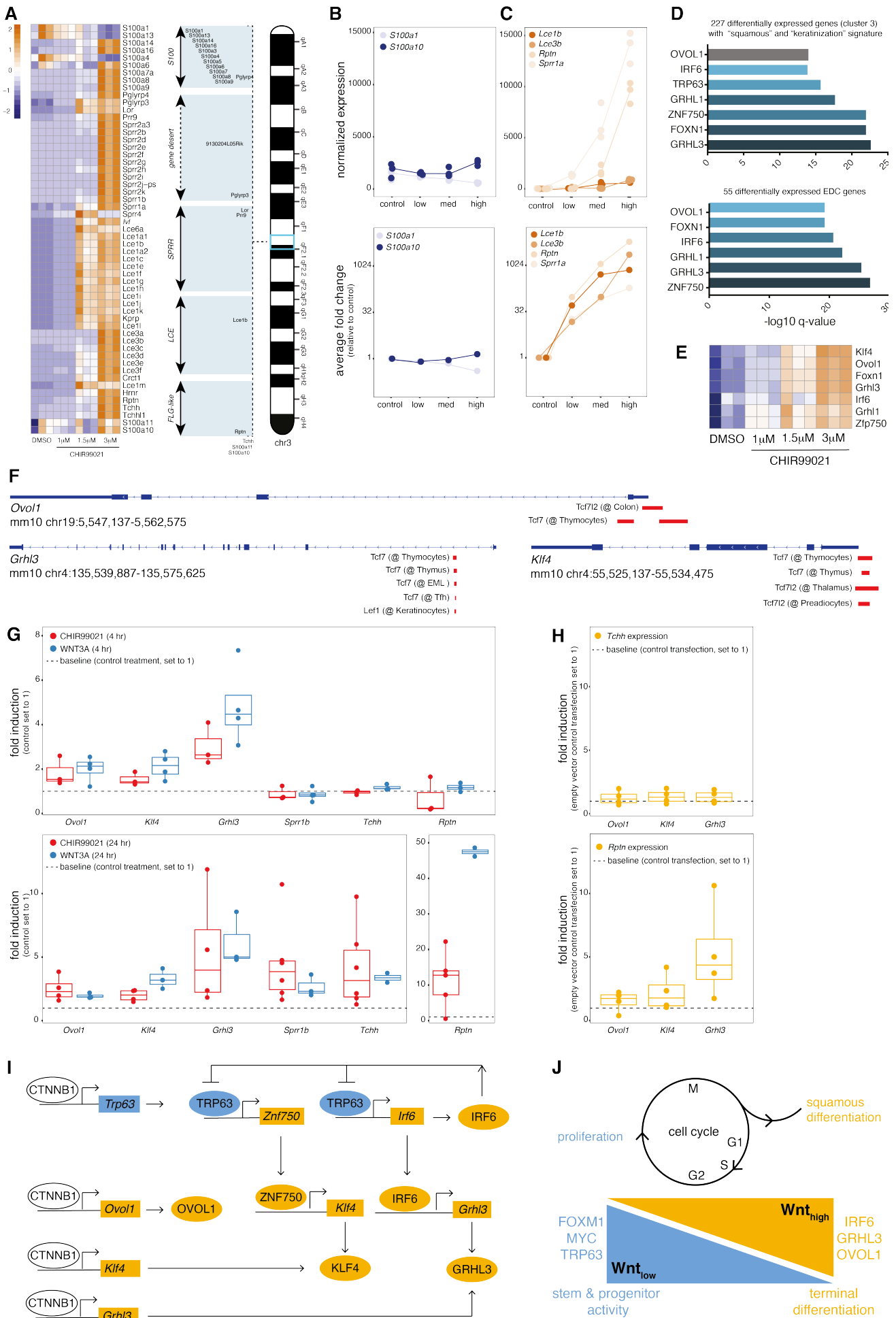
438



439 **Figure 3 High levels of WNT/CTNNB1 signaling induce squamous differentiation**

440 A-B) Heatmaps (unsupervised clustering, Z-score) showing gradual loss of A) a mammary stem cell signature and B)
441 myoepithelial features with increasing levels of WNT/CTNNB1 signaling. C-D) Graphs showing normalized expression values
442 of the three RNAseq replicates for C) two myoepithelial markers (*Otxr* and *Acta2*) and D) two basal markers (*Krt14* and
443 *Krt5*). E) Heatmap (unsupervised clustering, Z-score) showing gain of a squamous breast tumor signature with increasing
444 levels of WNT/CTNNB1 signaling. F) Brightfield microscopy image showing H&E staining of paraffin embedded organoids,
445 which reveals a core of keratinized material in the center of WNT_{high} organoids. G) Bar plot depicting the results of a gene
446 set enrichment analysis for genes that are differentially expressed in WNT_{low} (1 μ M CHIR99021) versus control (DMSO)
447 organoids. H) Graphs showing the average fold change in hair matrix (top: *Lef1*, *Ptch1*, *Shh*) and skin barrier markers
448 (bottom: *Flg*, *Krt1*, *Krt10*). Data points depict the mean of the three RNAseq replicates for each experimental condition,
449 with the control (DMSO) set to 1. I) Confocal microscopy image of an immunofluorescent staining of formalin fixed, paraffin
450 embedded sections of agarose-mounted organoid cultures on day 7. K5=KRT5 (basal marker), K8 = KRT8 (luminal marker),
451 K10 = KRT10 (suprabasal marker), LOR = Loricrin (terminal differentiation marker), DAPI = nuclei. Scalebar = 25 μ m
452 (overview) and 10 μ m (inserts). Source data provided in Supplementary File 3.

453



454 **Figure 4 WNT/CTNNB1 signaling induces master regulators of epidermal differentiation**

455 A) Heatmap (genes depicted in order of chromosomal location, Z-score) showing *de novo* expression of multiple EDC locus
456 genes. B-C) Graphs showing normalized expression (top) and average fold change (bottom) of two genes located at the
457 border (*S100a1* and *S100a10*) and four genes from the central region of the EDC locus (*Lce1b*, *Lce3b*, *Rptn*, *Sprr1a*). For
458 normalized expression values individual data points of the three RNAseq replicates are shown. For average fold change
459 values data points depict the mean of the three RNAseq replicates for each experimental condition. D) Bar plot depicting
460 the results of a gene set enrichment analysis for the 227 differentially expressed from cluster 3, which revealed the
461 keratinization signature (top) and for the 55 differentially expressed genes located in the EDC locus (bottom). E) Heatmap
462 (unsupervised clustering, log-transformed normalized expression values, Z-score) of 7 differentially expressed candidate
463 master regulatory transcription factors. F) Schematic depicting the results from ChIPseq analyses, revealing the presence
464 of common TCF/LEF binding sites (red blocks) close to the transcriptional start site of *Ovol1* (top), *Grhl3* (bottom left) and
465 *Klf4* (bottom right). G) Graphs depicting the results of quantitative RT-PCR analyses performed in BC44 cells, revealing the
466 induction of master regulators (*Ovol1*, *Klf4* and *Grhl3*) and a selection of EDC locus genes (*Sprr1b*, *Tchh* and *Rptn*) by both
467 CHIR99021 and purified WNT3A. Data points depict n=2-6 independent experiments, with each data point representing the
468 average of a technical triplicate. *Rpl13a* was used as a reference gene and all expression levels are plotted as fold increase
469 over control (DMSO or BSA treated cells). H) Graphs depicting the results of quantitative RT-PCR analyses performed in
470 BC44 cells, revealing the induction of *Rptn* (bottom) but not *Tchh* (top) following transient overexpression of GRHL3 and,
471 to a lesser extent, OVOL1 and KLF4. *Grhl3* and, to a lesser extent, *Ovol1* and *Klf4*. Data points depict n=4 independent
472 experiments, with each data point representing the average of a technical triplicate. *Rpl13a* was used as a reference gene
473 and all expression levels are plotted as fold increase over control (empty vector control transfected cells). I) Model of the
474 gene regulatory network that controls epidermal transdifferentiation. J) Model summarizing the WNT/CTNNB1-induced
475 competing proliferation and differentiation responses of the mammary epithelium. See text for details.

476 **SUPPLEMENTARY FILES AND SUPPLEMENTARY FIGURE LEGENDS**

477

478 **Supplementary File 1**

479 This file contains the output of the RNAseq analysis using DESeq2. It contains lists of differentially
480 expressed genes between the different treatment conditions (6 lists in total, FDR set at <0.1 for each)
481 as well as an overview of the normalized counts for all genes. The raw data for the RNAseq analysis
482 have been deposited in NCBI GEO (GSE178321).

483

484 **Supplementary File 2**

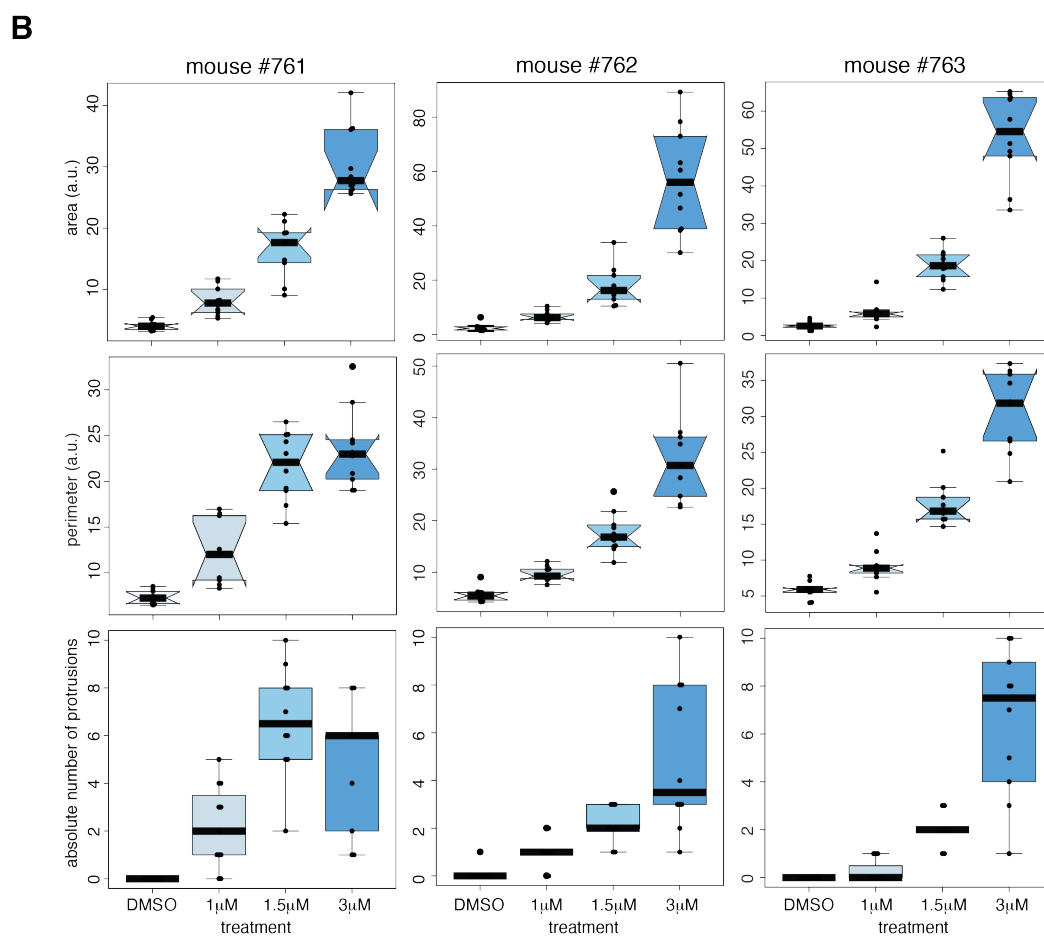
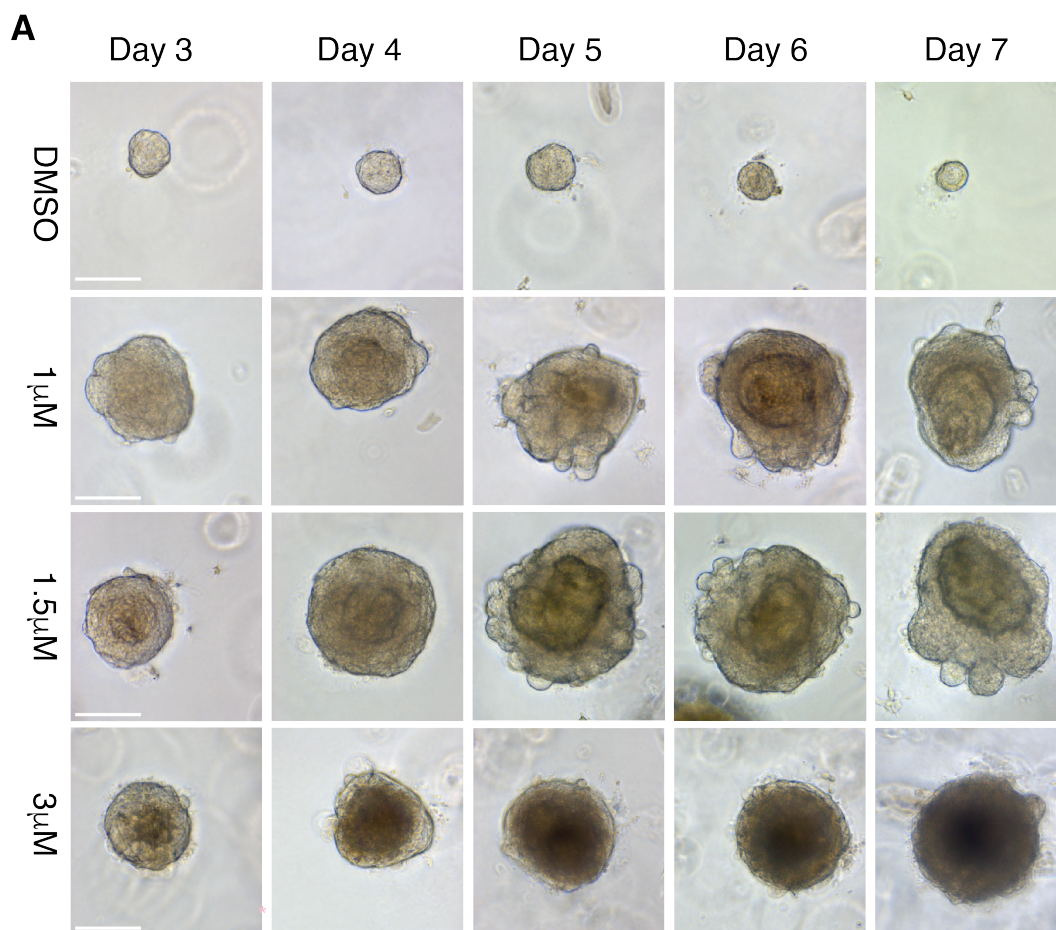
485 This file contains the gene lists that were used for the analyses mentioned in the text, and as depicted
486 in the main figures and Supplementary Figure 4. It also contains the raw results of the gene set
487 enrichment analyses.

488

489 **Supplementary File 3**

490 This file contains all of the source data and statistical analyses that were performed for the analyses
491 mentioned in the text and depicted in the main figures and Supplementary Figure 4.

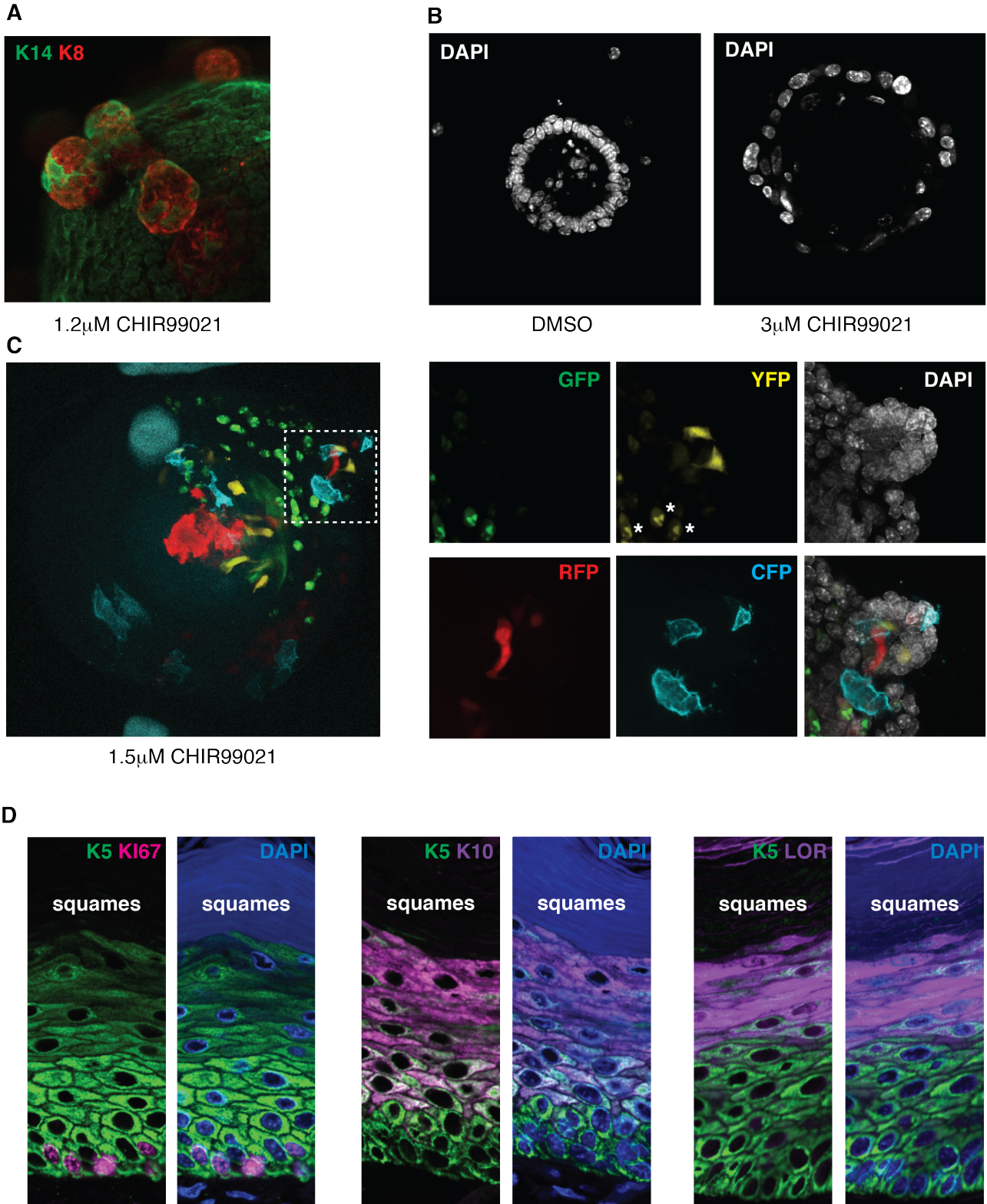
492



493 **Supplementary Figure 1**

494 A) Brightfield microscopy images showing temporal changes in size and morphology of a representative organoid culture.
495 B) Box plots showing changes in size (area, top row, and perimeter, middle row) and morphology (number of protrusions,
496 bottom row) in response to WNT/CTNNB1 hyperactivation. The three samples depicted (mouse #761, #762 and #763) are
497 the samples that were used for the RNAseq experiment. Images from #761 are depicted in 1H as “max” and images from
498 #762 are depicted in 1H as “min”. The RNAseq data deposited at NCBI GEO (GSE178321) are labelled “rep1” (for #763),
499 “rep2” (for #762) and “rep3” (for #761).
500

501

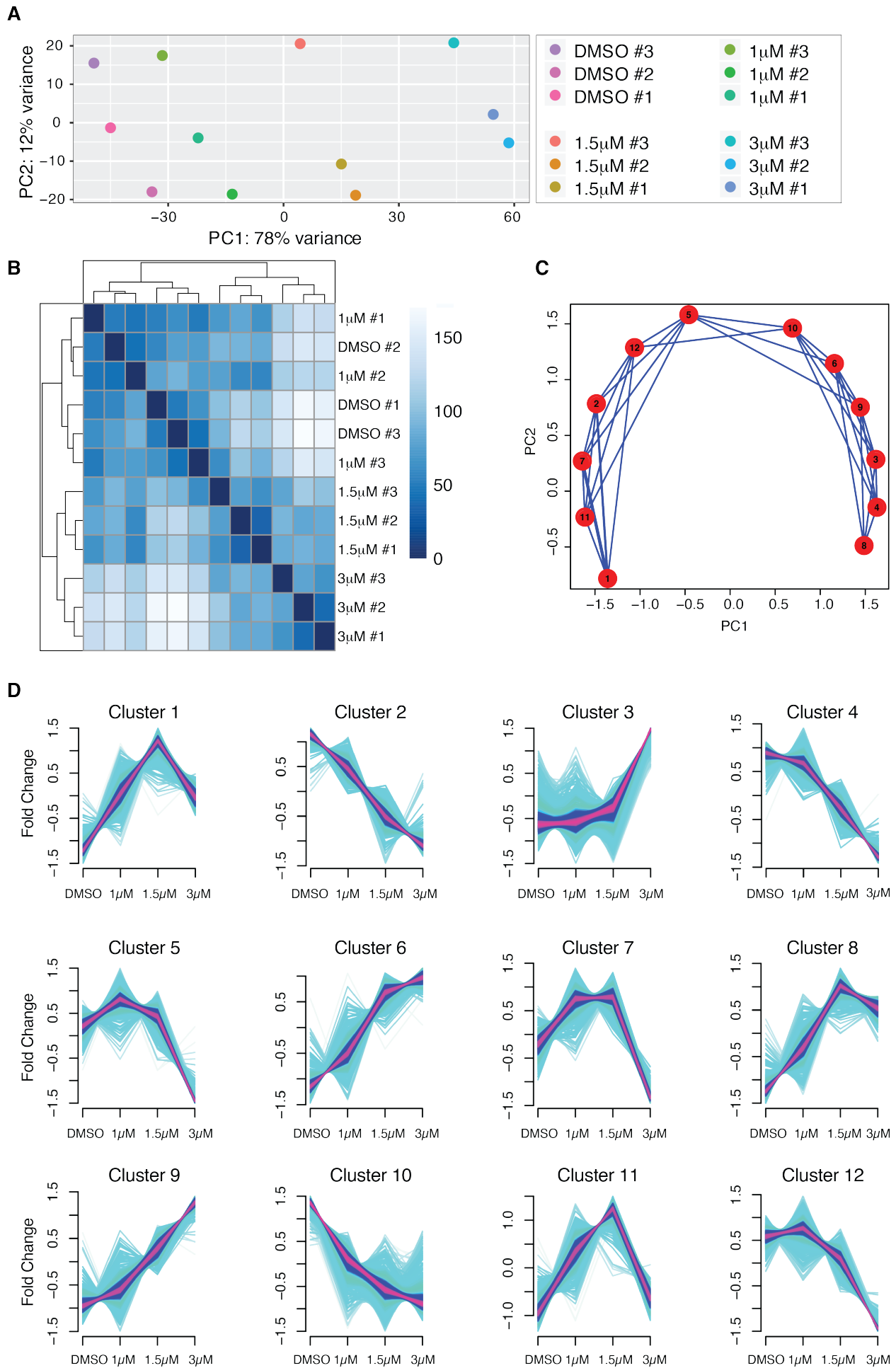


502

503

504 **Supplementary Figure 2**

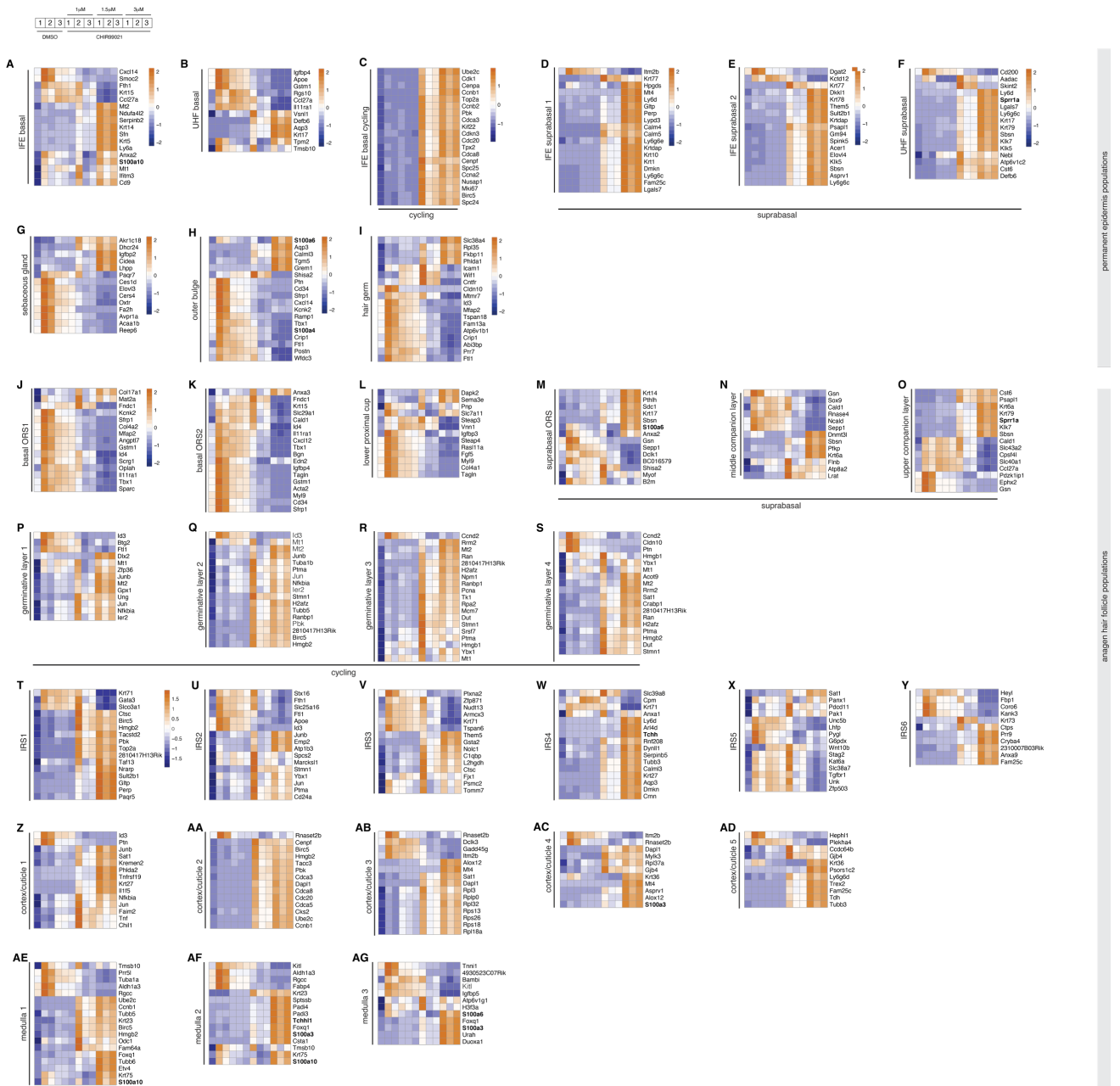
505 A) Wholemout confocal microscopy image showing protrusions of luminal cells (K8 = KRT8, luminal marker; K14 = KRT14,
506 basal marker). B) Wholemout confocal microscopy image showing a cross section of a representative control and a
507 representative WNT^{high} organoid, revealing changes in nuclear shape and size. Similar changes can be observed in main
508 Figures 1I, 2J and 3I. C) Wholemout confocal microscopy image showing lineage tracing of WNT/CTNNB1 responsive cells
509 in a WNT^{med} *Axin2*^{CreERT2}; *Rosa26*^{Confetti} organoid, revealing the outgrowth of multiple independent clones. Close ups of the
510 area in the dashed box are shown on the right. D) Confocal microscopy images of a stratified squamous epithelium (mouse
511 vagina), illustrating the pattern of cell division (KI67 signal in K5-positive basal cells) and differentiation (K10, suprabasal
512 cells, and LOR, flattened and cornified cells). Squames indicates the dead, keratinized material that also shows up as the
513 eosin stained core of the Wnt^{high} organoids in Figure 3F.



514 **Supplementary Figure 3**

515 A-B) Sample level quality control of the RNAseq analysis. A) Principle component analysis (PCA) plot for the three
516 independent RNAseq samples of the organoids depicted in Supplementary Figure 1B. B) Sample-level hierarchical
517 clustering. C-D) mFuzz cluster analysis. C) PCA plot for the 12 different clusters. D) Gene expression changes separating the
518 12 different clusters. The keratinization signature was picked up in cluster 3. Loss of the mammary stem cell signature was
519 picked up in cluster 2. This loss may seem counterintuitive, but it suggests that only low levels of WNT/CTNNB1 signaling
520 are able to promote mammary stem-cell fate. Loss of contractile features was picked up in cluster 10.

521



522 **Supplementary Figure 4**

523 Heatmaps (unsupervised clustering, Z-score) depicting differentially expressed genes of specific cell populations of the
 524 permanent epidermis (A-I) and the anagen hair follicle (J-AG). Gene lists were generated by taking the top 20 genes for each
 525 of the populations indicated from Supplementary Table S1 of Joost et al. (2020) Cell Stem Cell. Only genes that were
 526 differentially expressed in the organoid RNAseq data are depicted. A) Interfollicular epidermis (IFE) basal, B) Upper hair
 527 follicle (UHF) basal, C) IFE basal cycling, D) IFE suprabasal 1, E) IFE suprabasal 2, F) UHF suprabasal, G) sebaceous gland, H)
 528 outer bulge, I) hair germ, J) basal outer root sheath (ORS) 1, K) basal ORS2, L) lower proximal cup, M) suprabasal ORS, N)
 529 middle companion layer, O) upper companion layer, P-S) germinative layers 1 through 4, T-Y) inner root sheath (IRS) 1
 530 through 6, Z-AD) cortex/cuticle 1 through 5, AE-AG) medulla 1 through 3.

531

532 **MATERIALS AND METHODS**

533

534 **Resource Availability**

535

536 Further information and requests for resources and reagents should be directed to and will be fulfilled
537 by the lead contact, Renée van Amerongen (r.vanamerongen@uva.nl).

538

539

540 **Materials Availability**

541

542 Plasmids generated in this study have been deposited to Addgene:

543 pGlomyc-Grhl3 Addgene #172869

544 pGlomyc-Klf4 Addgene #172870

545 pGlomyc-Ovol1 Addgene #172871

546

547

548 **Data and code availability**

549

550 The RNAseq data generated during this study are available at NCBI GEO.

551 (GSE178321, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178321>)

552

553 All R packages used for bioinformatics analysis, data plotting and summary statistics are listed in the
554 materials and methods additional resources section.

555

556 All primer sequences are listed in the materials and methods additional resources section.

557

558 Source data for all graphs and heatmaps are available in the supplementary files.

559 EXPERIMENTAL MODEL AND SUBJECT DETAILS

560

561 Animals

562 All mice used for this study were maintained under standard housing conditions. Animals were housed
563 in open or IVC cages on a 12h light/dark cycle and received food and water *ad libitum*. All experiments
564 were performed in accordance with institutional and national guidelines and regulations and approved
565 by the Animal Welfare Committees of the University of Amsterdam and The Netherlands Cancer
566 Institute. All primary organoid cultures were established from FVB/NHan[®]Hsd mice (purchased from
567 Envigo), except for organoids used for lineage tracing, which were established from compound
568 *Axin2^{CreERT2};Rosa26^{Confetti}* animals (own colony, mixed background).

569 *Axin2^{CreERT2}* (Van Amerongen et al., 2012) and *Rosa26^{Confetti}* (Snippert et al., 2010) strains can be
570 obtained from Jackson labs (#018867: (B6.129(Cg)-Axin2tm1(cre/ERT2)Rnu/J; #017492: B6.129P2-
571 Gt(ROSA)26Sortm1(CAG-Brainbow2.1)Cle/J).

572

573 Primary mouse mammary organoid cultures

574 Mammary glands (third thoracic and fourth inguinal) were harvested from 8-11 week-old virgin female
575 mice. Mammary organoids cultures were established according to published protocols (Ewald et al.,
576 2008; Nguyen-Ngoc et al., 2015). Briefly, fat pads were minced with scissors (~ 20 times) and
577 transferred to a tube with 10 ml collagenase/trypsin solution consisting of DMEM/F12/Glutamax
578 (Gibco) supplemented with 0.02 g trypsin (Gibco), 0.02 g collagenase type IV (Sigma-Aldrich C5138), 5
579 ml Fetal Bovine Serum (Gibco), 250 µl of 1 µg/ml insulin (Sigma-Aldrich I6634) and 50 µl of 50 µg/ml
580 gentamicin (Sigma-Aldrich G1397), and were incubated for 30 min at 37°C shaking at 200 rpm. The
581 resulting suspension was centrifuged at 1500 rpm for 10 min, and then resuspended in 4 ml DMEM/F12
582 + 80 µl DNase (1U/µl) (Promega M6101). The DNase solution was gently shaken by hand for 2–5 min,
583 followed by centrifugation at 1500 rpm for 10 min. Four differential centrifugations (pulse to 1500 rpm
584 in 10 ml DMEM/F12) were performed to separate single cells (including fibroblasts) from organoids.
585 Isolated organoids were mixed with 50 µl of Growth Factor Reduced Matrigel (Corning), seeded in an
586 8 well chamber slide pre-coated with 20 µl of Matrigel per well, and incubated for 30 min at 37°C. After
587 matrigel polymerization, basic organoid growth media was added (DMEM/F12, 1% v/v insulin,
588 transferrin, selenium (Gibco 41400045) and 1% v/v penicillin/streptomycin (Gibco, 100X stock).
589 Organoids were cultured for 7 days in basic organoid medium and treated with either 3 µM, 1.5 µM or
590 1 µM CHIR99021 (BioVision/ITK Diagnostics; diluted in basic organoid medium from 6mM stock

591 resuspended in DMSO at 1:2000, 1:4000 and 1:6000, respectively) or DMSO (VWR, 1:1000) as vehicle-
592 control. Media was refreshed every 2-3 days and cultures were analyzed after 7 days.

593

594 **Cell lines**

595 The BC44 cell line (Deugnier et al., 1999) was a gift from Marie-Ange Deugnier (Institute Curie, Paris,
596 France). Cells were cultured in RPMI medium with L-Glutamine, 10% FBS and 5 µg/ml insulin and grown
597 at 37°C and 5% CO₂. For passaging, cells were trypsinized with 0.05% trypsin-EDTA for 5 minutes at
598 37°C, resuspended in medium and centrifuged for 4 minutes at 1500 rpm. The pellet was resuspended
599 in fresh medium and cells were counted using a Neubauer counting chamber. Cells were passaged 1:6
600 or 1:10 two to three times per week.

601

602

603 **METHOD DETAILS**

604

605 **Organoid embedding and sectioning**

606 Matrigel drops containing organoids were scooped from individual wells and incubated for 1.5 hours
607 in 800 µl Cell Recovery Solution (Corning) on ice. Samples were spun down for 4 minutes at 300 rcf at
608 4°C. After removal of supernatant, 500 µl 4% PFA was added to each sample and incubated for 1 hour.
609 Fixed organoids were spun down for 4 minutes at 300 rcf at 4°C and washed with Milli-Q water. The
610 organoid pellet was then embedded in 2% agarose (Sigma) followed by an overnight incubation in 70%
611 Ethanol. Agarose blocks containing organoids were sequentially incubated in 100% EtOH, 100%
612 isopropanol and terpene (Histoclear, National Diagnostics), each step at room temperature for 2 hours,
613 followed by incubation in liquid paraffin (Paraplast X-tra, Carl Roth, melting point: 50-54 °C) at 55°C.
614 Samples were embedded in paraffin and sectioned at 5 µm.

615

616 **Immunofluorescence**

617 Paraffin sections were incubated for 6 minutes in terpene (Histoclear, National Diagnostics) and
618 rehydrated in 100% isopropanol, following ethanol gradient baths (100%, 70% and 50%) and demi
619 water. For antigen retrieval, sections were heated for 2.5 hours at 85°C with 10mM sodium citrate (pH
620 6.0) solution. Sections were cooled to room temperature and incubated for 30 min in 0.3% H₂O₂ to
621 block endogenous peroxidase activity. After a PBS wash, sections were blocked for 1 hour with 2.5%
622 BSA and incubated with primary antibodies overnight. The following primary antibodies were used:
623 rat-α-K8 (1:250; TROMA-I; DSHB), rabbit-α-K14 (1:1000; PRB-155P; Biolegend), chicken-α-K5 (1:1000;

624 905901; Biolegend), rabbit- α -K10 (1:1000; 905404; Biolegend), rabbit- α -Loricrin (1:500; 905104;
625 Biolegend), rabbit- α -KI67 (1:100; ab16667; Abcam). Secondary antibodies were incubated in PBS for 1
626 hour at room temperature. The following secondary antibodies were used: goat α -rat Alexa Fluor 647
627 (1:1000; A21247; Invitrogen), goat α -rabbit Alexa Fluor 568 (1:1000; A11011; Invitrogen), goat α -
628 chicken Alexa Fluor 488 (1:1000; A11039; Invitrogen). Nuclei (DNA) were stained with 6-diamidino-2-
629 phenylindole dihydrochloride (DAPI, Invitrogen). Slides were embedded with Mowiol (0.33 g/ml
630 glycerol (Sigma-Aldrich 15523-1L-R), 0.13 g/ml Mowiol 4–88 (Sigma-Aldrich 81381-50G), 0.13M
631 Tris.HCL (pH 8.5)).

632

633 **H&E staining**

634 Paraffin-embedded sections were incubated for 60 min at 55 °C and de-paraffinized for 6 minutes in
635 terpene (Histoclear, National Diagnostics) and rehydrated in 100% isopropanol, following ethanol
636 gradient baths (100%, 70% and 50%) and demi water. Slides were stained with hematoxylin (Merck
637 Millipore) for 20-30 sec and then washed for 5 min in running tap water, following 3 min in PBS and 5
638 minutes in 70% Ethanol. Slides were stained with eosin (Sigma) for 3 min, washed twice in 70% EtOH
639 for 4 min, dehydrated in 70% EtOH, 100% EtOH, 100% isopropanol and terpene and sealed with a
640 coverslip using omnimount histological mounting medium (National Diagnostics).

641

642 **Lineage tracing**

643 4-hydroxytamoxifen (4-OHT, Sigma, #7904) was dissolved in 100% ethanol (1 mM stock solution). A
644 final concentration of 1 μ M 4-OHT was added to the organoid cultures on the day of plating (day 0).
645 On the next day, the media was replaced with media containing CHIR99021. Organoids were fixed with
646 4% PFA for 15 minutes on day 7, washed with PBS, washed with 0.15M glycine in PBS, washed in PBS
647 again, permeabilized with 0.5% Triton X-100 in PBS, washed in PBS, counterstained with TOPRO3 and
648 mounted with Vectashield.

649

650 **Microscopy**

651 Brightfield images of mammary organoid cultures were taken on a Zeiss Axio Vert.A1 phase contrast
652 microscope equipped with an AxioCam MRc. For imaging H&E stained slides, pictures were taken using
653 an Axioscope A1 microscope with a Nikon Ri2 camera and NIS F freeware. For imaging
654 immunofluorescence slides, pictures were taken using a Nikon A1 confocal microscope (20x water
655 immersion objective with an NA of 0.75) and NIS elements AR software. Excitation with 405nm (DAPI),
656 440nm or 458nm (CFP) 488nm (GFP or Alexa488), 514 nm (YFP), 561 nm (RFP or Alexa561) and 633 nm

657 (TOPRO3 or Alexa647) laser lines. For wholemount confocal imaging of fluorescently labelled
658 organoids, images were taken on a Leica SP5 or SP8 with AOBs.

659

660 **RNA sequencing**

661 Matrigel drops containing organoids were scooped from individual wells of an 8-well chamber slide
662 after 7 days of culture in the presence of either DMSO or CHIR99021 and lysed in 1 ml of Trizol Reagent
663 (Life Technologies). RNA extraction, purification, sequencing and data processing until read count
664 calculation were performed at the NKI Genomics Core facility as part of a collaboration with Dr. Jos
665 Jonkers. Briefly, RNA was extracted using the Qiagen RNeasy column purification kit. RNA quality was
666 checked with a Bioanalyzer (Agilent), after which polyA+ stranded RNA library preparation was
667 performed using the Illumina TruSeq stranded RNA prep kit. RNA-sequencing was performed on a
668 HiSeq 2500 (Illumina) System using a stranded protocol. Single-end reads (65 bp) were aligned to
669 reference sequence GRCm38/mm10 with Tophat version 2.1 and Bowtie version 1.1.0 (Trapnell et al.,
670 2009). Expression values were determined by HTSeq-count (Anders et al., 2015). Original .bam files and
671 raw counts have been deposited at NCBI GEO and are available under accession number GSE178321.

672

673 **Bioinformatics analysis**

674 Raw gene-level count tables were processed in R (R Core Team and Team, 2020), using DESeq2 (Love
675 et al., 2014). No pre-filtering was performed. Normalized values were extracted from the
676 DESeqDataSet (dds) object (provided in Supplementary File 1 as “annotated_normalizedcounts”).
677 Differentially expressed genes were extracted using pairwise comparisons of different treatment
678 conditions with $\text{padj} < 0.1$ as a cut off for false discovery (provided individually in Supplementary File
679 1). For Figure 2A each differentially expressed gene list was sorted according to the \log_2 fold change
680 and the top 50 activated and top 50 repressed genes were selected. The resulting gene lists were
681 combined, giving a total of 319 genes. The \log_2 transformed normalized expression values were used
682 to construct the heatmap depicted in Figure 2A.

683 Genes meeting the criteria for differential expression in one or more comparisons (11714 genes total,
684 provided in Supplementary File 1 as “normalized_values_DE_genes”) were used to extract gene lists
685 and plot heatmaps using the pheatmap package (Raivo Kolde, 2019). All heatmaps were made using Z-
686 score scaling and unsupervised clustering along rows, except for Figure 4A where genes are depicted
687 according to their chromosomal location. With the exception of Figure 2A, dendrograms were removed
688 in the final figures to save space.

689 To detect patterns of gene expression changes in our data, all genes that were differentially expressed
690 in one or more conditions were further analyzed in R using the Mfuzz package (Kumar and Futschik,
691 2007). Briefly, normalized readcounts were averaged per condition (DMSO, 1 μ M CHIR99021, 1.5 μ M
692 CHIR99021, 3 μ M CHIR99021) and the concentrations were converted to pseudotime (0, 10, 15, 30).
693 The fuzzifier m was estimated based on the expression set, which returned a value of 2.5. The optimal
694 number of clusters was determined empirically and set at 12 for the final analysis. Genes making up
695 the core of each cluster (based on a membership value >0.7) were extracted.

696 For gene ontology and gene set enrichment analyses, gene lists of interest (provided in Supplementary
697 Figure 2) were analyzed using “Investigate gene sets” at <http://www.gsea-msigdb.org> (Liberzon et al.,
698 2015; Subramanian et al., 2005). All 9 collections were queried: H (Hallmark gene sets), C1 (positional
699 gene sets), C2 (curated gene sets), C3 (regulatory target gene sets), C4 (computational gene sets), C5
700 (ontology gene sets), C6 (oncogenic signature gene sets), C7 (immunologic signature gene sets), C8 (cell
701 type signature gene sets). All gene set names showing specific enrichment are listed in Supplementary
702 File 2.

703 To find putative regulatory transcription factors, gene sets were analyzed using Enrichr
704 (<https://maayanlab.cloud/Enrichr/>) (Chen et al., 2013; Kuleshov et al., 2016). The following collections
705 were queried: ChEA 2016, ENCODE and ChEA Consensus TFs, ARCHS4 TFs Coexp, TF Perturbations. All
706 factors identified are listed in Supplementary File 3.

707 TCF/LEF ChIPseq data for mouse TCF7 (17 tracks total), TCF7L1 (1 track total), TCF7L2 (14 tracks total)
708 and LEF1 (4 tracks total) were downloaded from <https://chip-atlas.org/> and visualized in the IGV
709 browser (<https://software.broadinstitute.org/software/igv/>) (Robinson et al., 2011) aligned to mm10.

710

711 **DNA cloning**

712 PCR based cloning was used to amplify coding regions of candidate master regulator genes (*Ovol1*,
713 *Grhl3*, *Klf4*) from cDNA of BC44 cells treated with 3 μ M CHIR99021 for 4 hours. Primers were designed
714 with overhangs containing restriction enzyme sites of BamHI and EcoRI to enable restriction-enzyme
715 based cloning. PCR amplification was performed using Phusion High-Fidelity DNA Polymerase (2 U/ μ l;
716 Thermo Fisher, F-530L). For this 2 μ l of cDNA were mixed with 10 μ l of HF buffer, 5 μ l dNTPs, 2 μ l of
717 the forward primer and 2 μ l of the reverse primer, 0.5 μ l of Phusion, 1.5 μ l of DMSO and MQ sterile
718 water up to a final volume of 50 μ l. The PCR program used was the following: 95°C for 30s, followed by
719 34 cycles of 95°C for 10s, 55-72°C for 15s, 72°C for 60 s and a final incubation step at 72°C for 10
720 minutes.

721 The PCR product was checked on a 1% agarose gel for bands of the expected sizes (*Ovo1*= 800 bp,
722 *Grhl3* = 1800 bp, *Klf4* = 1452 bp). PCR products were purified using a GeneJET PCR purification kit
723 (Thermo Fisher) or a GeneJET gel extraction kit (Thermo Fisher) when purified from the agarose gel,
724 and digested with BamHI (ER0051, Thermo Fisher) and EcoRI (ER027, Thermo Fisher) for 2h at 37°C).
725 The pGlomyc3.1 vector (Van Amerongen et al., 2004; Jonkers et al., 1999) was digested with the same
726 enzymes (BamHI and EcoRI) for at least 4 hours at 37°C. After digestion, Alkaline Phosphatase (AP)
727 treatment was performed on digested vector to prevent recircularization during ligation, adding 1 µl
728 of FastAP enzyme (Thermo Fisher, EF0654) and 2 µl of 10X FastAP buffer ($c_f = 1X$) (Thermo Fisher, #B64)
729 and incubating it at 37°C for 10-15 min.
730 Digested PCR products and digested vector backbone were checked on a 1% agarose gel and purified
731 using a GeneJET gel extraction kit (Thermo Fisher) or purified directly from the mix without running it
732 on the gel using a GeneJET PCR purification kit (Thermo Fisher). Purified digested PCR product and
733 digested dephosphorylated vector were ligated together for 2 hours at room temperature using a 1:1
734 and a 1:3 vector:insert ratio. Transformation of the ligated products was performed by mixing 5 µl of
735 DNA with 25 µl of DH5α competent *Escherichia coli* cells. A vector only control (digested and
736 dephosphorylated) was used as a negative control. A mixture of DNA and bacteria was incubated on
737 ice for 15 min, heat shock was performed in a 42°C water bath for 1 min and the mixture was returned
738 to ice. Afterwards, 250 µl of Lysogeny broth (LB) was added to each transformation and incubated at
739 37°C for 30 min. After incubation, 100 µl of the mixture was plated on Ampicillin (Amp) LB agar plates
740 and incubated overnight at 37°C. From each condition, 12 single colonies were picked. Plasmid DNA
741 was purified from miniprep cultures using a GeneJET plasmid DNA miniprep kit (Thermo Fisher, K0502).
742 Constructs were checked with a control digestion performed using the same restriction enzymes used
743 for ligation (BamHI and EcoRI). Samples containing DNA bands of the expected sizes for insert (*Ovo1*=
744 800 bp, *Grhl3* = 1800 bp, *Klf4* = 1452 bp) and vector (~ 6 Kb) were sequenced verified. One miniprep of
745 each construct cloned was selected based on sequencing results and maxipreps were prepared using
746 a GeneJET Plasmid DNA Maxiprep kit (Thermo Fisher, K0491) to obtain a high yield of plasmid DNA.
747 Sequencing revealed no mutations, except for pGlomyc-Klf4, where a silent mutation was found in
748 codon 133 (CCG → CCA, proline).

749

750 **BC44 cell treatment and transfection**

751 For the experiments depicted in Figure 4F, cells were treated with 3 µM CHIR99021 (DMSO as a vehicle
752 control) or 50 ng/ml purified Wnt3a (RnD) (BSA as a vehicle control) for 4 or 24 hours prior to
753 harvesting. For the experiments depicted in Figure 4G, BC44 cells were plated in a concentration of

754 150,000 cells/well in 6-well plates and transfected the next day with 2 µg of the designed plasmids and
755 X-tremeGENE HP DNA Transfection Reagent (Sigma) using a 1:1 ratio of µl X-tremeGENE HP DNA
756 Transfection Reagent to µg DNA. First, DNA was diluted in Opti-MEM reduced serum media (GIBCO) to
757 a final concentration of 1 µg plasmid DNA/100 µl medium (0.01 µg/µl); then X-tremeGENE HP DNA
758 Transfection Reagent (1 µl/µg DNA) was vortexed and added without touching the walls of the
759 Eppendorf tube; the mix was incubated for 20 minutes at room temperature. Cell culture media was
760 refreshed before adding the transfection mix in a dropwise manner. Empty pGlomyc_3.1 vector was
761 transfected as a negative control. Cells were harvested 24 hours after transfection.

762

763 **cDNA synthesis and qRT-PCR**

764 BC44 cells were lysed in 1 ml TRIzol (Invitrogen) and processed according to the manufacturer's
765 instructions. Briefly, the cells were lysed in the tissue culture plate for 1 minute after which the lysate
766 was transferred to an Eppendorf tube and incubated at RT for another 5 minutes. Next, 200 µl of
767 chloroform was added to the RNA lysates. Tubes were vortexed briefly and incubated at RT for 3
768 minutes. Samples were then centrifuged for 15 minutes (12,000g at 4°C). The aqueous phase was
769 transferred to new tubes, after which 500 µl of isopropanol and 1 µl of glycogen were added. Tubes
770 were vigorously shaken every minute for 10 minutes total, after which they were centrifuged for 10
771 minutes (12,000g at 4°C). The RNA pellet was washed twice by removing the supernatant, adding 1 ml
772 of 75% ethanol, vortexing briefly and centrifuging 5 for minutes (12,000g at 4°C). After the second wash
773 step, the ethanol was taken off and the pellet was left to air dry. The RNA was dissolved in 20 µl RNase
774 free water and heated for 15 minutes at 55°C. RNA concentrations and purity were measured using a
775 NanoDrop.

776 To normalize RNA input for qPCR analysis 2 µg of RNA was used for cDNA synthesis. Reverse
777 transcription was performed using the SuperScript IV First-Strand Synthesis System (ThermoFisher
778 Scientific), according to manufacturer's protocol. Random hexamer primers were used for reverse
779 transcription. Briefly, RNase free water was added to 2 µg of RNA until a volume of 14 µl was reached.
780 To this mix, 4 µl of 5x SSIV buffer and 2 µl of DNase (RQ1, Promega) was added. The samples were then
781 incubated for 30 minutes at 37°C. After this, 2 µl DNase stop solution was added and the samples were
782 incubated again, this time for 10 minutes at 65°C. After incubation, 2 µl random hexamer primers and
783 2 µl dNTPs were added to each sample. Samples were then incubated for 5 minutes at 65°C. After
784 incubation, the samples were stored on ice until cold. To each sample, 8 µl RNase free water, 4 µl 5x
785 SSIV buffer, 2 µl DTT and 0.2 µl RiboLock enzyme was added. Samples were mixed and 20 µl was
786 transferred to new tubes. 1 µl of SuperScript IV Reverse Transcriptase (RT) was added to the new tubes.

787 No enzyme was added to the remaining mix (-RT control). Both the +RT and -RT mixes were incubated
788 for 10 minutes at 23°C, 10 minutes at 55°C, and lastly 10 minutes at 80°C. The cDNA was then diluted
789 with 180 µl RNase free water.

790 Per sample, a mix was made containing 10 µl RNase free water, 4 µl 5x HOT FIREPol EvaGreen qPCR
791 Supermix (Biotium), 0.5 µl forward (10 µM stock) and 0.5 µl reverse primer (10 µM stock). This was
792 pipetted into 96-well plates (0.2 ml), after which 5 µl cDNA was added with clean filter tips. The plate
793 was covered with MicroAmp adhesive film and centrifuged for 5 minutes at 1500 rpm.

794 Quantitative PCR reactions were run on a QuantStudio 3 (Applied Biosystems) using the following
795 program: Hold stage (2 minutes on 50°C, 15 minutes on 95°C), PCR stage (15 seconds on 95°C, 1 minute
796 on 60°C, 40 cycles) and the Melt Curve stage (15 seconds on 95°C, 1 minute on 60°C, 1 second on 95°C).

797 For every experiment, *Rpl13a* was used as a reference gene. Real-time PCR quantification of gene
798 expression was performed in triplicate with one -RT control for each cDNA sample and primer pair.

799 To calculate relative expression of the genes of interest, the comparative quantification ($\Delta\Delta Ct$) method
800 was used. To this end, the mean Ct value of the technical triplicate values was calculated for all samples.

801 *Rpl13a* mean Ct values were used for normalization and vehicle control treated samples were used as
802 a calibrator in each experiment. To compare different experiments, all vehicle controls were set to 1
803 and fold changes over vehicle control were calculated for treated samples. Individual qPCR
804 experiments were analyzed in Microsoft Excel. Data from n=2-6 individual experiments (performed by
805 two independent experimenters) were pooled for the final graphs in Figure 4.

806

807

808 **Primer sequences**

809 The following primers were ordered from IDT:

<i>qRT-PCR analysis</i>		
Gene	Forward (5' to 3')	Reverse (5' to 3')
<i>Axin2</i>	AGCTGGTTGTACCTACT	CAGGCAAATTCGTCCTCG
<i>Grhl3</i>	CAAGGAAGATGACCTTCAGAGAG	CAACATGAGCGCGTCAAA
<i>Klf4</i>	CGGGAAGGGAGAAGACT	GAGTTCCTCACGCCAACG
<i>Ovol1</i>	CTCCACGTGCAAGAGGAACT	CTCTGGTCCCCTAGGG
<i>S100a14</i>	ATGGGACAGTGTCCGGTCAG	GTGTCTCAATGGCCTCTCT
<i>Sprr1b</i>	ACACTACCTGTCTCCATATACCAG	TGTTTCACTTGTTGCTCATGC
<i>Rpl13a</i>	CCCTCCACCTATGACAAGA	GCCCCAGGTAAGCAAATT
<i>Rptn</i>	CCCATGATAGAAGGGAGCAG	GGTTGTCCTTTAGGCTTCC
<i>Tchh</i>	AAGCGAGATGGTCAATACCTG	CCTGGCGACGAATCGTAT
<i>DNA cloning</i>		
Gene	Forward (5' to 3')	Reverse (5' to 3')
<i>Ovol1</i>	5'-AAAAAGGATCCTATGCCTCGCGGTTCTG-3'	5'-AAAAAGAATTCTCAGAGATGGGGGCTGCC-3'
<i>Grhl3</i>	5'-TAAGCAGGATCCGATGTCGAATGAACTTGATTC-3'	5'-GAAGAGAATTCTCATAGCTCCTCAGGATGATC-3'
<i>Klf4</i>	5'-AAAAAGGATCCTATGAGGCAGCCACTGGC-3'	5'-GGGGAGAATTCTAAAAGTGCCTTTCATGTG-3'
<i>genotyping primers</i>		
Strain	Primer sets	Expected bands
<i>Axin2^{CreERT2}</i>	RVA282: GCACGTTACCGGCATCAAC RVA109: AAGCTGCGTCGGATACTTGAGA RVA110: AGTCCATCTTCATTCCGCTAGC	WT: 500 bp MUT: 800 bp
<i>Rosa26^{Confetti}</i>	RVA340: GAATTAATTCCGGTATAACTTCG RVA341: AAAGTCGCTCTGAGTTGTTAT RVA342: CCAGATGACTACCTATCCTC	WT: 386 bp MUT: 300 bp

810

811

812

813 **QUANTIFICATION AND STATISTICAL ANALYSIS**

814

815 All details (including exact value and definition of n, fold-changes/effects sizes, confidence intervals,
816 etc.) can be found in the figure legends, main text or supplementary file 3.

817

818 No statistical methods were used to determine strategies for randomization or sample size estimation.

819

820 **Organoid measurements (Figure 1 and Supplementary Figure 1)**

821 A total of n=22 independent experiments (i.e. organoid cultures from individual mice) were analyzed.

822 For every condition, 9-44 organoids were photographed and used to calculate area, perimeter and

823 circularity in Fiji. For each image, the number of protrusions was counted by hand. Per condition, the

824 9-44 data points were averaged into a single number, resulting in n=22 data points total for each

825 measurements. Mean or median numbers were plotted. Statistical testing was performed in R. Source

826 data and summary statistics are provided in Supplementary File 3. Effect sizes for Figure 1G were

827 calculated using Plots of Differences (Goedhart, 2019).

828

829 **Organoid quantification (Figure 2)**

830 To obtain the total cell number and K8-positive cells for each condition, the DAPI and K8 channels for

831 each organoid image were loaded separately into CellPose (Stringer et al., 2021). Images were

832 calibrated and segmented based on the nuclear or cytoplasm model, respectively. If necessary, the

833 model thresholding was edited manually for each individual image to achieve optimal segmentation.

834 The segmentation of each image was saved as a mask image that can be imported to ImageJ as ROIs

835 via the LabelstoROIs plugin (Waisman et al., 2021). The total number of ROIs for the DAPI channel

836 corresponds to the total number of cells present in each image, and the total number of K8 ROIs

837 corresponds to the total number of luminal cells. The total K8 negative population, which contains both

838 cells of a mammary and an epidermal cell fate, is obtained by subtracting the number of K8 ROIs from

839 the DAPI ROIs. The ki67 signal was thresholded manually in FIJI and converted to a binary (dividing/non-

840 dividing) signal. Before counting the ki67+ cells, either the DAPI or K8 ROIs were eroded by 1 pixel to

841 exclude overlap between individual cells and to minimize the cytoplasm in K8 ROIs. The Ki67 signal in

842 the DAPI and K8 ROIs was subsequently measured. All measurements were Excel. The total number of

843 ROIs with a positive (255) median signal were counted to determine the number of ki67 positive cells.

844 Images from n=2 (DMSO) or n=3 (all other conditions) independent organoid cultures were counted.

845 Because different cell numbers were counted for each experiment and experimental condition, the

846 data were averaged into a single data point for each individual experiment. Source data and statistics
847 are provided in Supplementary Figure 3.

848

849 **ADDITIONAL RESOURCES**

850

851 **Software**

852 Data were handled and analyzed in Microsoft excel and R (packages: ggplot2, ggpubr, dplyr,
853 ggbeeswarm, tidyr, rstatix, rcompanion, Rmisc, DescTools, Boot, DESeq2, Biobase, RColorBrewer,
854 pheatmap, Mfuzz). All heatmaps and graphs were made in R Studio or Prism, except for Figure 1G
855 which was made using the online Plots of Differences tool. Microscopy images were analyzed using Fiji
856 and multi-channel overlays were made using the Image 5D plugin. All final figures were composed in
857 Adobe Illustrator.

858

859 **Databases and online tools**

860 Plots of Differences: <https://huygens.science.uva.nl/PlotsOfDifferences/>

861 CellPose: <http://www.cellpose.org>

862 MSigDB: <https://www.gsea-msigdb.org/gsea/msigdb/>

863 Enrichr: <https://maayanlab.cloud/Enrichr/>

864 ChIP atlas: <http://chip-atlas.org>

865 IGV browser: <https://software.broadinstitute.org/software/igv/>

866

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872

873 **Competing interests**

874 The authors declare that no competing interests exist.

875

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