

HPV8-induced STAT3 activation led keratinocyte stem cell expansion in human actinic keratoses

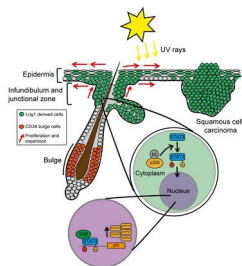
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1 **HPV8-induced STAT3 activation led keratinocyte stem cell expansion in**
2 **human actinic keratoses**

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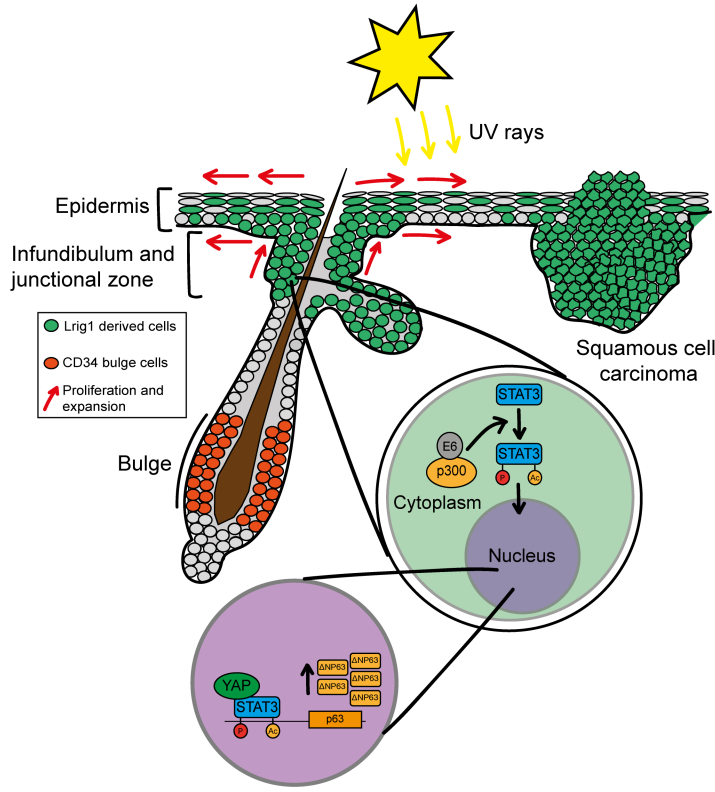
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HPV8 reactivation in actinic keratoses



38 **Abstract**

39 Despite epidermal turnover, the skin is host to a complex array of microbes including
40 viruses, such as the human papillomavirus (HPV), which must infect and manipulate skin
41 keratinocyte stem cells (KSC) to survive. This crosstalk between the virome and KSC
42 populations remains largely unknown. Here, we investigated the effect of HPV8 on KSCs
43 using various mouse models. We observed that the HPV8 early region gene E6 specifically
44 caused Lrig1⁺ hair follicle junctional zone KSC proliferation and expansion, which would
45 facilitate viral transmission. Within Lrig1⁺ KSCs specifically, HPV8 E6 bound intracellular
46 p300 to phosphorylate the STAT3 transcriptional regulatory node. This induces Δ Np63
47 expression, resulting in KSC expansion into the overlying epidermis. HPV8 was associated
48 with 70% of human actinic keratoses (AK). Together these results define the “hit and run”
49 mechanism for HPV8 in human actinic keratosis as an expansion of KSCs, which lacks
50 melanosome protection and is thus susceptible to sun-light-induced malignant
51 transformation.

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64 **Introduction**

65 Human skin hosts a microbiota that has maintained symbiosis through evolution. Integral to
66 this environmental interface is a large and diverse array of viruses, the virome, which is
67 capable of manipulating host cellular processes to reside as symbionts (1–3). As obligate
68 intracellular parasites, notably *Papillomaviridae* have to infect long-lived cells such as skin
69 keratinocyte stem cells (KSC) to withstand constant epidermal turnover (4). Among the
70 various skin KSC populations, the hair follicle (HF) KSCs have been implicated as host cells
71 for *Beta-papillomaviruses* (5–7). Recently, HF KSCs have been identified as important
72 regulators in the crosstalk between the bacterial microbiome and the immune system(8–
73 11). The virome is unaffected by antibiotics, yet similar to the bacterial microbiome,
74 expansion, and diversification are observed with impaired immunity. Relative to healthy
75 individuals, subtle shifts in immune function such as in epidermodysplasia verruciformis
76 (EV) or dedicator of cytokinesis 8 (DOCK8) deficiency, directly alter the virome leading to
77 increased diversity and expanded representation of skin tropic β and γ human
78 papillomavirus (HPV) types, which in turn increase the risk of skin cancer (12–15). Similarly,
79 alterations in virome have been observed among solid organ transplant recipients on
80 immunosuppression, who exhibit a 60-250-fold increased risk of skin cancer (16–22). As
81 such, there is a clinical imperative to elucidate the crosstalk between HPV and host KSCs.

82 The *Papillomaviridae*, HPV family, are small (7-8 kb) non-enveloped DNA viruses that
83 undergo episomal replication within differentiating keratinocytes. HPV contains three distinct
84 coding regions: (1) an upstream regulatory region, (2) an early region with typically up to six
85 open reading frames (E1, E2, E4, E5, E6 and E7), and (3) a late region encoding capsid
86 proteins L1 and L2 (23, 24). Tissue tropism is determined by the L1 protein, with negatively
87 charged L1 protein of β and γ HPV types selectively targeting human non-mucosal skin,
88 while the positively charged L1 protein on α HPV types result in mucosal infection (24, 25).
89 Micro-abrasion facilitates viral entry into basal keratinocytes, and therein KSCs, in order to

90 establish long-term infection, wherein E1 and E2 proteins support replication of the viral
91 genome at a low copy number. It is only when these infected basal cells differentiate, thus
92 moving closer to the surface, that the viral load increases to support viral transmission (26).
93 In the majority, HPV infection is an asymptomatic infection that may result in transient warts
94 or keratoses, countered by the immune response that blocks viral replication. However,
95 persistent infection with high-risk HPV types, notably α HPV, is associated with cancer
96 accounting for an estimated 600,000 cases per annum (27). In α HPV, viral integration into
97 the host DNA is postulated to deregulate expression of E6 and E7 proteins leading to
98 keratinocyte transformation, however, transformation can also occur without integration
99 (28). In the complex structure of the skin with appendageal structures, high-risk β HPV
100 (HPV5, 8, and 38) have been associated with pre-malignant skin changes, called actinic
101 keratoses (AK), which in association with ultraviolet (UV) light exposure, risk transformation
102 to cutaneous squamous cell carcinoma (cSCC) (16–18, 29–36). However, β HPV DNA
103 integration is not observed, and the viral load in ensuing cSCC is low, leading authors to
104 postulate a “hit and run” mechanism for transformation (30, 37).

105 Consistent with the potential oncogenic role of high-risk β HPV, FVBN transgenic mice
106 expressing HPV8 early region genes under the control of the keratin 14 (Krt14) promoter
107 (HPV8-CERtg) exhibit skin changes mirroring human AK and spontaneously develop cSCC,
108 which occur with greater frequency after UV light exposure (38, 39). HPV8-CERtg mice
109 crossed with Rag 2 deficient mice, to recreate the immunosuppressive tumor
110 microenvironment similar to that observed in organ transplant recipients on
111 immunosuppressants, demonstrated accentuated tumor growth (40). In addition, cSCC has
112 also been observed in transgenic mice expressing individual HPV8 early region genes E2,
113 E6 and E7 under the control of the Krt14 promoter (41–43). Recently we identified that the
114 HF junctional zone KSC (herein denoted as JZSC) population, defined by the expression of
115 leucine-rich repeats and immunoglobulin-like domains protein 1 (Lrig1) on the cell surface,

116 was selectively expanded in the HPV8-CERTg mice (38). In this manuscript, we elucidate
117 the crosstalk between HPV8 and KSCs, redefining the basis for AK, and thus identify HPV8
118 to be a major risk factor for human cSCC.

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135 **Results**

136 The HF contains multiple KSC populations that under homeostatic conditions maintain
137 keratinocyte numbers during hair cycling, but also retain the capacity to regenerate the
138 whole HF (Figure 1A). To determine how HPV8 selectively drives the proliferation and
139 expansion of the Lrig1⁺ JZSC population we utilized the HPV8-CERTg mouse model,
140 wherein the Krt14 promoter regulates the expression of the HPV8 early region genes in
141 basal cells of the entire epithelium (Supplemental Figure 1A). Immunofluorescent labeling of
142 skin with antibodies binding to the basal keratin, Krt14 and the differentiation marker
143 involucrin showed that the HPV8-CERTg compared to wild type (WT) had an expansion of
144 the undifferentiated keratinocytes within the infundibulum and overlying epidermis, with a
145 reduction in the involucrin:Krt14 ratio (Figure 1B). To confirm that Lrig1⁺ JZSC proliferation
146 led to expansion into the infundibulum and overlying epidermis, we crossed
147 Lrig1CreER^{T2}:R26RConfetti and Krt15CrePGR:R26RConfetti mice with HPV8-CERTg mice
148 (Figure 1C). The Confetti mouse contains a four-color cassette, which recombines within
149 individual cells upon Cre activation to express one of four fluorescent proteins: green
150 (GFP), red (RFP), yellow (YFP) or cyan (CFP) (44). In WT Lrig1CreER^{T2}:R26RConfetti and
151 Krt15CrePGR:R26RConfetti mice, lineage-labeled progeny as expected remained
152 compartmentalized to the infundibulum and sebaceous gland (Lrig1CreER^{T2}:R26RConfetti
153 mice) or to the lower hair follicle and inner root sheath (Krt15CrePGR:R26RConfetti mice)
154 (Figure 1D). Likewise Krt15CrePGR:R26RConfetti:HPV8-CERTg mice showed a similar
155 distribution of labeled cells compared to their WT counterpart. However,
156 Lrig1CreER^{T2}:R26RConfetti:HPV8-CERTg mice showed fluorescent clones extending into
157 the infundibulum and perifollicular epidermis (Figure 1D). Thus, HPV8-induced selective
158 proliferation of the Lrig1⁺ JZSC population resulted in the expansion of this population into
159 the infundibulum and perifollicular epidermis.

160 **c-MYC transcriptional regulation distinguishes JZSC from HF bulge KSC**

161 **during homeostasis**

162 Lrig1⁺ JZSCs are transcriptionally distinct from the lower HF bulge KSC populations, which
163 are typically characterized by CD34 cell surface expression (38, 45, 46). To determine why
164 HPV8 induced selective proliferation of the Lrig1⁺ JZSCs, but not other KSCs, we undertook
165 a transcriptomic analysis of these adjacent HF KSC populations defined by Lrig1 and CD34
166 expression in adult mice. Both Lrig1⁺ and CD34⁺ flow-sorted keratinocytes were isolated
167 from dorsal back skin of individual mice, using established protocols, for pairwise
168 comparison (Figure 1E). Illumina HiSeq4000 paired-end sequenced samples resulted in a
169 total of 35,566,700 reads, of which 47% mapped to 54,658 murine genes (GRCm38).
170 Principal-component analysis distinguished both genotype and KSC population
171 transcriptomes (Figure 1F). Unsupervised hierarchical clustering of Log₂ transformed,
172 median centred, average linkage by Pearson's correlation showed primary segregation of
173 KSC populations with the influence of HPV8 as a secondary separation (Supplemental
174 Figure 1B), suggesting that the regulatory signaling pathway networks between adjacent
175 KSC populations are largely distinct and independent of the effects of HPV8 early region
176 genes. Normalization of count data and DESeq2 pipeline analysis, identified 3029
177 differentially expressed genes (DEGs; $p < 0.05$) from Lrig1⁺ vs CD34⁺ flow-sorted
178 keratinocytes in WT mice (Figure 1G and Supplemental Table 1). These DEGs were
179 enriched for 330 genes that distinguish Lrig1⁺ from CD34⁺ KSC populations previously
180 identified by microarray analysis (Supplemental Figure 1C)(46). Gene set enrichment
181 analysis (GSEA)(47, 48) of the DEGs determined that the Lrig1⁺ JZSCs were involved in
182 upper-hair follicle and sebaceous gland differentiation (positive enrichment) and were
183 distinct from the CD34⁺ outer layers (negative enrichment) of the hair follicle bulge
184 keratinocytes (Supplemental Figure 1D). GSEA identified c-Myc as the central regulating
185 node that distinguished these two KSC populations, in WT and HPV8-CERtg, with Lrig1⁺
186 JZSC flow-sorted keratinocytes showing a greater enrichment of the *c-Myc* signature when

187 compared with the CD34 population, consistent with the role of the JZSC in maintaining
188 sebaceous gland sebocyte differentiation (Figure 1H). Thus, Lrig1 JZSCs under
189 homeostatic conditions represent a distinct HF KSC population distinguished by activation
190 of *c-Myc*.

191 Transcriptomic analysis of flow-sorted Lrig1⁺ and CD34⁺ KSC populations from adult HPV8-
192 CErTg mice yielded 1427 DEGs, of which 46% overlapped with DEGs from similar analysis
193 in WT mice (654 genes); consistent with the primary segregation found in our unsupervised
194 hierarchical clustering. Bioinformatic analysis of HPV8-CErTg flow-sorted cells
195 demonstrated findings that were similar to WT mice, but there was no further enrichment of
196 *c-Myc* to account for increased proliferation from GSEA (Figure 1I), nor difference in
197 transcript counts or *c-Myc* gene expression by qPCR (Figure 1J). Therefore, *c-Myc*
198 activation differentiates Lrig1⁺ and CD34⁺ KSC populations in both WT and HPV8-CErTg
199 HFs but was not responsible for the HPV8-induced proliferation of only the Lrig1⁺ JZSC
200 population.

201 **STAT3 activation drives Lrig1 JZSC proliferation**

202 While the bioinformatic analysis comparison of Lrig1⁺ and CD34⁺ HF KSC populations
203 yielded a total of 3802 DEGs (Figure 1G), in contrast, there were a total of 276 DEGs ($p <$
204 0.05) from the analysis of HPV8-CErTg vs WT for the two HF KSC populations (Figure 2A).
205 There were only two shared genes, β -1,4-galactosyltransferase 6 and olfactory receptor
206 family 12 subfamily D member 3, suggesting that the transcriptional impact of HPV8 early
207 region genes was unique to individual KSC populations even when closely situated,
208 consistent with the observed increased proliferation of the Lrig1⁺ JZSC but not the CD34
209 bulge KSC population.

210 We hypothesized that HPV8 must selectively activate a growth factor pathway(s) in the
211 Lrig1⁺ JZSCs but not the CD34 bulge KSC population for there to be selective proliferation.
212 Therefore, we utilized Ingenuity Pathway Analysis canonical pathways comparative

213 software package (Qiagen), inputting all DEGs (adjusted $p < 0.05$, Supplemental Table 1)
214 from comparisons of HPV8 Lrig1 vs WT Lrig1 and HPV8 CD34 vs WT CD34. Only the
215 STAT3 signaling canonical pathway demonstrated activation and reached significance ($p <$
216 0.05) with differential expression in the Lrig1⁺ HPV8 vs WT when compared to the CD34⁺
217 HPV8 vs WT analyses (Supplemental Figure 1E). There was greater phosphorylated
218 STAT3 observed within hair follicle keratinocytes from HPV8-CERTg mouse skin
219 (Supplemental Figure 1F). Although glucocorticoid receptor, HIF1- α , and Hippo signaling
220 pathways also demonstrated differential expression, these did not reach significance.
221 GSEA of the STAT3 gene signature in the Lrig1 HPV8 vs Lrig1 WT mouse DEGs
222 demonstrated a normalized enrichment score of 1.1, whereas the CD34 HPV8 vs CD34 WT
223 comparison was only 0.6 (Figure 2B). Nuclear labeling of phosphorylated STAT3 was
224 evident within the hair follicle junctional zone, infundibulum and adjoining interfollicular
225 epidermis of HPV8tg skin, consistent with its role in driving Lrig1⁺ JZSC proliferation and
226 expansion (Figure 2C). Western blot analysis of HPV8-CERTg and WT mouse back skin
227 keratinocytes identified similar levels of full-length and transcriptionally active STAT3 α
228 (86kDa) as the predominant splice variant (Figure 2D). Consistent with activation of the
229 STAT3 pathway, HPV8-CERTg mouse skin nuclear fractions demonstrated 2.2-fold increase
230 in Tyr705 STAT3 phosphorylation (within the transactivation domain), but no change in
231 Ser727 STAT3 phosphorylation (within the COOH terminus) (Figure 2E). STAT3
232 downstream transcriptionally regulated genes were increased in expression by qPCR in
233 HPV8-CERTg Lrig1⁺ JZSCs relative to the CD34⁺ bulge KSC population (Figure 2F).

234 To determine if the STAT3 regulatory node was essential for HPV8-induced Lrig1⁺ JZSC
235 proliferation, we crossed HPV8-CERTg mice with Krt5Cre-Stat3^{+/flox} mice to generate HPV8-
236 CER:STAT3^{+/-}tg mice, since STAT3 knockout is known to be embryologically lethal. We
237 have previously shown that HPV8-CER:STAT3^{+/-}tg mice had WT levels of Tyr705 STAT3
238 phosphorylation, and demonstrated a four-fold reduction in tumor formation (49). Confocal
239 laser scanning microscopy (CLSM) imaging using IMARIS™ 3D rendering software of

240 fluorescently labeled Lrig1⁺ JZSCs in whole mount tail skin, showed no expansion in HPV8-
241 CER:STAT3^{+/-}tg mice (Figure 2G). Consistent with the lack of KSC proliferation, $\Delta Np63$
242 expression levels in HPV8-CER:STAT3^{+/-}tg were comparable to those in WT skin (Figure
243 2H), specifically also in the Lrig1⁺ flow-sorted cells (Figure 2I). Similarly, $\Delta Np63$ expression
244 levels in the HPV8-CER:STAT3^{+/-}tg flow-sorted CD34⁺ bulge KSCs remained unchanged
245 (Supplemental Figure 1G). In summary, HPV8-induced Lrig1⁺ JZSC proliferation was
246 dependent upon STAT3 Tyr705 phosphorylation.

247 **HPV8 E6-induced proliferation of Lrig1 JZSC**

248 To determine which of the HPV8 early region protein(s) was responsible for STAT3
249 activation and therefore specific proliferation of the Lrig1⁺ JZSC population, we compared
250 the adult mouse skin HFs from WT, HPV8-CERtg and those expressing the individual early
251 region genes: HPV8-E2tg, HPV8-E6tg and HPV8-E7tg (Figure 3A). Anagen HF lengths
252 were similar between WT and the different mouse genotypes: WT ($418.20 \pm 11.25 \mu\text{m}$),
253 HPV8-CERtg ($414.38 \pm 9.01 \mu\text{m}$), HPV8-E2tg ($412.26 \pm 8.44 \mu\text{m}$), HPV8-E6tg
254 ($429.56 \pm 1.85 \mu\text{m}$) and HPV8-E7tg ($430.43 \pm 13.31 \mu\text{m}$) ($n > 100$ HFs per genotype in 3 mice
255 per genotype) (Figure 3A). The expanded infundibulum area observed in the HPV8-CERtg
256 (4.67 ± 0.33 cells) compared to WT (2.33 ± 0.33 cells) was also observed in the mice with
257 individual early region genes E2 (3.13 ± 0.29 cells) and E6 (4.56 ± 0.29 cells), but not E7
258 (2.67 ± 0.33 cells) (Figure 3A). Next, we fluorescently labeled Lrig1⁺ JZSCs and used CLSM
259 with IMARIS™ 3D rendering software of whole mount tail skin from mice expressing
260 individual early region genes, HPV8-CERtg and WT (Figure 3B). Compared to WT
261 ($168.79 \pm 9.06 \mu\text{m}^3$) the Lrig1⁺ population volume in HPV8-CERtg ($273.517 \pm 33.89 \mu\text{m}^3$) and
262 HPV8-E6tg alone ($248.94 \pm 12.86 \mu\text{m}^3$) were significantly larger (Figure 3B). In contrast,
263 HPV8-E2tg ($194.70 \pm 7.54 \mu\text{m}^3$) and HPV8-E7tg ($152.30 \pm 15.49 \mu\text{m}^3$) demonstrated no
264 significant increase in the Lrig1⁺ JZSC population. The CD34⁺ bulge KSC population was
265 unchanged in the transgenic mice from that observed in WT mice (Supplemental Figure
266 2A). Proliferation, assessed by ki67 immunofluorescent labeling, was significantly greater in

267 the Lrig1⁺ JZSC in HPV8-CERTg (15.00±4.51%) and HPV8-E6tg (14.50±2.56%) compared
268 to WT (7.60±4.08%), whereas no increase was observed in: HPV8-E2tg (8.00±4.34%),
269 and HPV8-E7tg (6.88±2.10%) (Figure 3B and Supplemental Figure 2B). As expected, there
270 was no increase in proliferation within the CD34⁺ HF bulge KSC population compartment in
271 the transgenic mice compared to that in WT mice (Supplemental Figure 2A).

272

273 The Lrig1⁺ JZSC population was quantified by flow cytometry in WT and transgenic mouse
274 dorsal back skin keratinocytes labeled with Lrig1 and CD34. In contrast to the unchanged
275 CD34⁺ population, the Lrig1⁺ population was greater in HPV8-CERTg (5.85±1.32%) and
276 HPV8-E6tg (6.84±0.80%) transgenic mice compared to WT (3.05±0.65%), HPV8-E2tg
277 (1.98±0.56%) and HPV8-E7tg (1.13±0.32%) (Figure 3C and Supplemental Figure 2B).

278 Flow-sorted Lrig1⁺ keratinocytes from transgenic and WT mice did not express *Cd34*, and
279 similarly had low expression levels of other HF KSC markers *Lgr5* and *Lgr6* (Figure 3D).

280 Consistent with KSC proliferation and enrichment, the Lrig1 population from HPV8-CERTg
281 and HPV8-E6tg mice exhibited increased expression of $\Delta Np63$ relative to WT mice (Figure
282 3E) and reduced expression of differentiation-associated *Krt10* (Figure 3F). The reduction in
283 *Krt10* expression was not observed in the CD34 population from the same mice when
284 compared to WT (Supplemental Figure 2C). Flow-sorted Lrig1⁺ keratinocytes demonstrated
285 a four-fold increase in colony-forming efficiency (CFE) in the HPV8-E6tg Lrig1⁺ population
286 (0.24±0.11%) compared to WT (0.05±0.04%) (Figure 3G). Consistent with the E6-driven
287 Lrig1⁺ JZSC proliferation and expansion, the flow-sorted Lrig1⁺ JZSCs retained elevated
288 expression of *Sox9* and *c-Myc* compared to CD34⁺ KSCs (Figure 3H and Supplemental
289 Figure 2D). HPV8-E6tg mouse tissue sections had increased KSC proliferation-associated
290 proteins YAP and P63 (Figure 3I). Thus, the HPV8 E6 alone was sufficient to cause HPV8-
291 induced Lrig1⁺ JZSC proliferation and expansion.

292

293 **HPV8 E6 induces Lrig1⁺ JZSC expansion into the overlying epidermis**

294 HPV8-E6tg mouse skin nuclear fractions demonstrated 2.7-fold greater Tyr705 STAT3
295 phosphorylation, but no change in Ser727 STAT3 phosphorylation (Figure 4A). Next, we
296 sought to determine whether HPV8 E6-driven Lrig1⁺ JZSC proliferation was sufficient to
297 cause expansion into the overlying epidermis by lineage tracing. We crossed
298 Lrig1CreER^{T2}:R26RConfetti and Krt15CrePGR:R26RConfetti mice with HPV8-E6tg mice to
299 generate Lrig1CreER^{T2}:R26RConfetti:HPV8-E6tg and Krt15CrePGR:R26RConfetti:HPV8-
300 E6tg mice, wherein recombination occurred in nearly all cells (98%; data not shown). As
301 before, Lrig1CreER^{T2}:R26RConfetti:HPV8-E6tg demonstrated epidermal fluorescence;
302 consistent with Lrig1⁺ JZSC proliferation and expansion into the overlying epidermis (Figure
303 4B). Confetti labeled cells could be identified by flow-cytometry in the GFP channel using
304 the 488nm laser, enabling us to simultaneously identify Lrig1⁺ cells by antibody labeling,
305 such that we could isolate flow-sorted Lrig1⁺ confetti⁺ and their Lrig1⁻ confetti⁺ progeny from
306 Lrig1CreER^{T2}:R26RConfetti:WT and Lrig1CreER^{T2}:R26RConfetti:HPV8-E6tg (Figure 4C).
307 As expected there were more Lrig1⁻ confetti⁺ progeny in the
308 Lrig1CreER^{T2}:R26RConfetti:HPV8-E6tg compared to Lrig1CreER^{T2}:R26RConfetti:WT. WB
309 analysis of nuclear fractions demonstrated 2-fold greater Tyr705 STAT3 phosphorylation in
310 Lrig1⁺ confetti⁺ and Lrig1⁻ confetti⁺ from Lrig1CreER^{T2}:R26RConfetti:HPV8-E6tg compared
311 to Lrig1CreER^{T2}:R26RConfetti:WT (Figure 4D). RNA sequencing of flow-sorted populations
312 showed that a relatively small number of DEGs (533) differentiated Lrig1⁺ confetti⁺ from
313 Lrig1CreER^{T2}:R26RConfetti:HPV8-E6tg and Lrig1CreER^{T2}:R26RConfetti:WT mice (Figure
314 4E and Supplemental Figure 3A; Supplemental Tables 2 and 3), but more than twice as
315 much (212) as in the reciprocal comparison between HPV8-CERtg and WT mice (Figure
316 2A); suggesting that the other early region genes may mitigate against the effect of E6.
317 Consistent with the expansion and migration of Lrig1⁺ JZSC into the overlying epidermis,
318 GSEA identified STAT3, EMT (Figure 4F) and stem cell proliferation, but not differentiation
319 or MYC (Supplemental Figure 3B) gene signatures in the comparison of the Lrig1⁺ confetti⁺
320 populations. A larger number of DEGs (6087) distinguished the Lrig1⁻ confetti⁺ populations,

321 wherein GSEA similarly identified STAT3, EMT but not differentiation (Figure 4G), as well
322 as stem cell and migration (Supplemental Figure 3C) gene signatures in the HPV-E6tg
323 population. Surprisingly, GSEA of HPV8-E6tg Lrig1⁺ confetti⁺ vs Lrig1⁻ confetti⁺ identified
324 negative enrichment for STAT3, but no difference in EMT gene signatures, suggesting that
325 STAT3 transcriptional node activation was still evident within the Lrig1 progeny (Figure 4H).
326 Consistent with STAT3 activation in the Lrig1⁺ JZSC and their progeny in HPV8-E6tg, there
327 was a large overlap in DEGs when these populations are compared to WT (Supplemental
328 Figure 3D). Comparison of HPV8-E6tg Lrig1⁺ confetti⁺ and Lrig1⁻ confetti⁺ demonstrated
329 similar expression levels of STAT3-regulated genes compared to their WT counterparts
330 (Figure 4I). Consistent with retained KSCs within the HPV8-E6tg Lrig1⁺ JZSC progeny, we
331 determined no difference in CFE between HPV8-E6tg Lrig1⁺ confetti⁺ and Lrig1⁻ confetti⁺
332 keratinocytes (Figure 4J). Hence, HPV8-E6 induced selective proliferation of Lrig1⁺ JZSC
333 that led to expansion of KSCs into the overlying infundibulum and epidermis.

334 **E6 bound P300 activates the STAT3 regulatory node**

335 To determine how the HPV8 E6 protein might activate the STAT3 regulatory node, we used
336 the previously reported stably transduced human keratinocytes, HaCaT (Figure 5) and PM1
337 (Supplemental Figure 4)(50, 51). Consistent with our findings in the HPV8-CERtg and
338 HPV8-E6tg mouse keratinocytes, but in contrast to vector control and E7, the E6-
339 transduced HaCaT cells demonstrated higher levels of pSTAT3 Y705 when looking at
340 nuclear protein fractions (Figure 5A). Likewise, E6-transduced HaCaT cells, but not E7-
341 transduced HaCaT cells, exhibited increased expression of STAT3 downstream target
342 genes; including $\Delta Np63$ (Figure 5B and Supplemental Figure 4A). When cultured
343 keratinocytes were subjected to higher calcium concentrations to simulate epidermal
344 differentiation, E6-transduced cells maintained $\Delta Np63$ expression and exhibited delayed
345 expression of the differentiation marker involucrin, compared to vector control and E7-
346 transduced cells (Figure 5C). There was no difference in proliferation (Figures 5D and
347 Supplemental Figure 4B), however, HPV8 E6-transduced keratinocytes demonstrated 1.5-

348 fold greater CFE when compared to vector control and E7 (Figure 5E and Supplemental
349 Figure 4C). Consistent with the egress of HPV8 Lrig1⁺ JZSCs, E6-transduced human
350 keratinocytes migrated significantly faster when compared to vector only and E7-transduced
351 cells (Figure 5F and Supplemental Figure 4D). Therefore, E6-transduced human
352 keratinocytes demonstrated activation of the STAT3 regulatory node, which regulates the
353 expression of $\Delta Np63$, increasing KSC and migratory potential.

354 Across several HPV genotypes E6 binding partners have been identified using
355 immunoprecipitation and mass spectroscopy, of which HPV8 E6 bound 7 proteins: EP300
356 (P300), CREB binding protein, SMAD3, LRP1, LRRC15, MAML1 and NOTCH1 (52).
357 String™ analysis identified two related histone acetyltransferase proteins that regulate
358 transcription via chromatin remodeling and also acetylate STAT3, thereby enhancing its
359 transcriptional activity: P300 (Combined Score:0.986) and CREB binding protein (Combined
360 Score:0.967)(53–60) (Figure 5G and Supplemental Figure 4). HPV8 E6 has a relatively
361 unique 132-136 amino acid sequence that directly facilitates binding to the ubiquitously
362 expressed related paralog transcriptional co-activators P300 and CREB binding protein
363 (61). A single amino acid substitution, HPV8 E6 K136N, could block P300 binding and in
364 transgenic mice expressing this mutant E6 prevent papilloma formation after UVB exposure
365 (62). Here we show that HPV8 E6 K136N mutation did not induce STAT3 Y705
366 phosphorylation (Supplemental Figure 4E) nor increase $\Delta Np63$ expression (Supplemental
367 Figure 4F). Furthermore, mice expressing HPV8 E6 K136N demonstrated normal levels of
368 Tyr705 STAT3 phosphorylation compared to native HPV8 E6 (Supplemental Figure 4G).
369 P300 was ubiquitously expressed in HPV8 mouse keratinocytes (Figure 5H) and
370 transduced human keratinocytes (Figure 5I and Supplemental Figure 4H). Knockdown of
371 P300 expression by siRNA, decreased STAT3 Y705 phosphorylation and $\Delta Np63$
372 expression (Figure 5J, Supplemental Figure 4K). Likewise, knockdown of STAT3 also led to
373 a reduction in $\Delta Np63$ expression (Figure 5K, Supplemental Figure 4L). Total STAT3
374 immunoprecipitation of nuclear extracts showed a greater amount of acetylated STAT3 in

375 E6 compared to vector control (Figure 5L). Consistent with these findings, STAT3 chromatin
376 immunoprecipitation confirmed enriched binding of endogenous STAT3 to the putative
377 STAT3-responsive element within the 5'-flanking region Δ Np63 promoter in E6-transduced
378 cells relative to vector control cells (Figure 5M). Hence, HPV8 E6 binding to P300 is
379 necessary for activation of the STAT3 regulatory node.

380 **YAP contributes to STAT3 regulated Δ Np63 expression.**

381 KSCs demonstrate nuclear YAP translocation, as diminished Hippo signaling leads to
382 unphosphorylated YAP translocating into the nucleus where it can interact with STAT3 to
383 participate in transcription (63). To determine if nuclear YAP was essential for HPV8 E6-
384 induced Lrig1⁺ JZSC proliferation, we first determined that nuclear YAP was increased in
385 nuclear protein extracts from Lrig1⁺ flow sorted HPV8-E6tg vs WT mouse skin and E6-
386 transduced HaCaT cell line by Western blot (Figure 6A and B, respectively). YAP siRNA
387 knockdown in E6-transduced HaCaT cells, but not vector control cells, led to a reduction in
388 Tyr705 STAT3 phosphorylation (Figure 6C and Supplemental Figure 5, respectively). As
389 nuclear translocation of YAP has previously been reported when cells are cultured sparsely,
390 we similarly observed nuclear YAP within sparsely cultured E6-transduced HaCaT cells,
391 relative to vector control, wherein it co-localized with Tyr705 STAT3 phosphorylation and
392 Δ Np63 expression (Figure 6D). Furthermore, immunoprecipitation of YAP nuclear protein
393 extracts from E6-transduced HaCaT cells grown at ~50% confluence demonstrated higher
394 levels of bound STAT3 and Δ Np63 compared to vector control, suggesting that YAP may
395 be a co-transcription factor for both STAT3 and Δ Np63 (Figure 6E). Knockdown of YAP
396 reduced HPV8 E6 induced cell proliferation (Figure 6F). Hence, HPV8-E6tg KSC
397 proliferation was dependent upon YAP as an essential co-factor for STAT3 and Δ Np63
398 transcription.

399 **HPV8 is associated with human actinic keratoses**

400 HPV8 has been linked with keratoses and cSCC in patients with the primary
401 immunodeficiency syndrome EV, wherein koilocytes are present, and in other forms of
402 immunosuppression. Thus, we hypothesized that the presence of koilocytes in AK may
403 indicate HPV8 reactivation within the general population, from immunosuppression
404 associated with aging and/or sun exposure. Of 275 patients with pathologist-defined AK, we
405 determined the presence of koilocytes in haematoxylin and eosin-stained tissue samples in
406 193 (70%) (Figure 7A). Using a representative subset of 77 cases (44 with koilocytes), we
407 determined that the presence of koilocytes in AK was not associated with significant
408 differences in age, sex (Fisher's exact test, NS), body location or histological classifications
409 (Supplemental Table 4). To determine the presence of HPV8, we used a β -HPV L1 open
410 reading frame PCR-reverse hybridisation assay (detects 25 β -HPV types), DNA analysis by
411 nested PCR and tissue immunofluorescence for HPV8 E4 protein (Supplemental Figure
412 6A). The PCR-reverse hybridisation assay, which has been reported to be the most
413 sensitive assay (64), identified 6 of 43 HPV8 positive samples with a high yield (>100 DNA
414 copies per cell) and 37 of 43 HPV8 positive samples with low yield (no HPV47 was
415 detected). The presence of koilocytes within AK was 100% sensitive for HPV8 with a 98%
416 positive predictive value. In the remaining koilocyte AK case, HPV38 was detected,
417 whereas in the absence of koilocytes in AK no β -HPV types were detected (Figure 7A).
418 Thus, AK koilocytes predicted the detection of HPV8. Consistent with HPV8 E7-mediated
419 ubiquitination and proteasomal degradation (Supplemental Figure 6B), AK samples with
420 koilocytes had lower levels of Rb1 protein (Figure 7B), even though p16 expression showed
421 no difference (Supplemental Figure 6C). We observed a much greater frequency of nuclear
422 pSTAT3 Y705 labeling within the epidermis of koilocyte containing AK than without,
423 $23.10 \pm 3.38\%$ vs $7.68 \pm 2.92\%$, respectively (Figure 7C). In normal skin, p63 antibody
424 labeling identified basal cells, but in AK, suprabasal p63 labeling was observed in the HF
425 infundibulum and adjoining epidermis (Figure 7D). The frequency of p63 labeling was

426 marginally greater among AK with koilocytes (Figure 7D). Although the detection of HPV8
427 DNA does not necessarily infer viral reactivation, the presence of koilocytes in AK
428 associated with reduction in Rb1 is highly suggestive.

429 Human epidermal keratinocytes are protected from UV-induced DNA damage by
430 melanosomes transferred from adjacent melanocytes, which forms a “melanin” cap over the
431 nucleus. We therefore hypothesized that the constant proliferation and translocation of
432 JZSCs into the adjoining epidermis may lessen melanosome protection. Compared to
433 normal skin, melanin staining was absent in all AK, irrespective of the presence of
434 koilocytes (Figure 7E). Thus, since the absence of melanosomes is common, next we
435 studied the DNA damage response to DNA double-strand breaks, which involves
436 phosphorylation of the histone variant H2AX at serine 139 in the flanking regions of
437 chromatin and can be labeled with specific antibodies that form visible foci in mammalian
438 cells (65). The percentage of nuclei with phosphorylated H2AX labeling was much greater
439 in AK with koilocytes than without, $92.43 \pm 3.97\%$ vs $53.86 \pm 8.40\%$, respectively
440 (Supplemental Figure 6D). UV frequently mutates p53 in human AK, as expected there was
441 no difference in the frequency of nuclear p53 in AK, with ($41.10 \pm 6.36\%$) and without
442 ($36.46 \pm 5.34\%$) koilocytes in the epidermis (Figure 7F); similarly, there was no difference in
443 p21 labeling (Supplemental Figure 6E). Hence based on the HPV8-CERtg mouse model, in
444 human AK with koilocytes, HPV8 may also activate pSTAT3 to drive p63 expression leading
445 to HF junctional zone expansion and displacement without melanin protection into the
446 overlying UV-exposed epidermis (Supplemental Figure 6F).

447

448

449

450 **Discussion**

451 The mammalian skin contains several adult tissue stem cell populations, wherein the Lrig1⁺
452 JZSC represents a transcriptionally distinct population (45, 66). Although Lrig1 expression
453 itself has been used to identify a number of adult stem cell populations in different tissues
454 (46, 67–71). Lrig1 is a negative regulator of epidermal growth factor receptor (EGFR)
455 signaling and therefore promotes stem cell quiescence by binding to EGFR, causing its
456 ubiquitination and proteasomal degradation (72, 73). Furthermore, EGFR signaling
457 activates the c-Myc transcriptional node resulting in Lrig1 expression, such that Lrig1
458 expression represents an autoregulatory negative feedback loop (46, 72, 74). Loss of Lrig1
459 expression in the skin leads to autonomous JZSC proliferation with increased cell numbers
460 in the overlying HF infundibulum and perifollicular epidermis (46, 75). During homeostasis,
461 as shown in our Lrig1CreER^{T2}:R26RConfetti model, the Lrig1⁺ JZSC population contributes
462 to the maintenance of cell numbers in the sebaceous gland and infundibulum (68, 76, 77).
463 In keeping with this, it has also been proposed that Lrig1 JZSC transformation is the basis
464 for sebaceous carcinoma (78). Thus, during homeostasis, the Lrig1⁺ JZSCs represents a
465 tightly regulated distinct functional HF population.

466

467 Here we have shown that HPV8 early region genes, which are shared by other HPV across
468 the genera, specifically circumvent the Lrig1⁺ JZSC c-Myc regulatory node to induce
469 proliferation and KSC expansion into the interfollicular epidermis. The c-Myc transcriptional
470 node clearly distinguishes the Lrig1⁺ JZSCs from the CD34⁺ HF bulge KSC population,
471 even in the context of HPV8 early region gene expression. Herein we describe an
472 alternative pathway for Lrig1⁺ JZSC proliferation governed by the STAT3 regulatory node
473 signaling through downstream target genes, which include *c-Myc* and $\Delta Np63$. This
474 pathway, which is only activated in the Lrig1 JZSC population, is driven by HPV8 E6 protein
475 interaction with P300, which activates STAT3. Importantly, we show that STAT3 activation

476 causes the proliferation and expansion of KSCs through increased symmetric cell division,
477 allowing KSCs to be displaced from their niche into the interfollicular epidermis. Moreover, it
478 appears that Lrig1 is an important, but pleiotropic factor that inhibits STAT3 and multiple
479 other growth factor receptors from signaling; including c-Met (79), RET (80), neurotrophic
480 receptor tyrosine kinase 2 (TrkB, NRTK2)(81), TNF α (82), and androgen receptors (83).
481 Corneal wounding, in the absence of Lrig1, led to STAT3 activation and premature corneal
482 opacification (84). Whether STAT3 regulatory node activation in the Lrig1 JZSC population
483 is responsible for the transient transfer of KSCs into the interfollicular epidermis after injury,
484 since epidermal loss of STAT3 is associated with delayed wound healing, remains to be
485 determined (85–87). In the context of HPV, this mechanism may allow the virus to reside in
486 a protected JZSC population and upon reactivation still release virions via the overlying
487 interfollicular epidermis.

488

489 STAT3 activation, which is associated with cytokine signaling in immune cells, has been
490 observed in several malignancies, both within cancer cells, but also, within the tumor
491 microenvironment immune cells (88). Numerous oncogenic signaling pathways converge to
492 give rise to constitutive STAT3 activation, although less frequent STAT3 oncogenic
493 mutations occur in myeloproliferative and skin malignancies (89). Inhibitors of the IL-
494 6/JAK/STAT3 pathway are already in clinical use, and novel STAT3 selective inhibitors are
495 currently in development. Constitutive activation of STATs, in particular STAT3, is found in
496 carcinoma from the head and neck (90), lung (91), breast (92), ovary (93), and prostate
497 (94). Within the context of cSCC, STAT3 deficiency is sufficient to block tumor formation in
498 the two-step chemical skin cancer mouse model, wherein tumors develop from within the
499 HF bulge KSC population (95). Although not described in the context of the Lrig1 JZSC
500 population, constitutive expression of activated STAT3 in the skin leads to keratinocyte
501 proliferation and expansion, similar to what we have observed in the

502 Lrig1CreER^{T2}:R26RConfetti:HPV8-CERtg but not in the Krt15CrePGR:R26RConfetti:HPV8-
503 CERtg mice, with increased susceptibility for UV-induced transformation (96). Consistent
504 with the importance of STAT3 signaling in HPV8, HPV8-CER:STAT3^{+/-}tg mice did not
505 demonstrate Lrig1⁺ JZSC expansion nor tumorigenesis.

506

507 HPV8 E6 exhibits intrinsic oncogenic activity (43), but unlike α HPV it does not bind and
508 inactivate p53 by rapid proteasome-mediated degradation; although it may prevent its
509 stabilisation (97, 98). Multiple studies have demonstrated the ability of HPV8 to bind p300
510 (52, 61, 62, 99, 100). The ability of HPV8 E6 to transform keratinocytes has been studied
511 for other binding partners impacting tumor suppressor and oncogenic pathways: Notch
512 (101–103), TGF β (104), Hippo (105), EGFR (106) and Wnt (107). In addition to the ability of
513 HPV8 E6 bound p300 to activate the STAT3 pathway, as described herein, the association
514 has been shown to attenuate activation of two essential DNA repair kinases ATM and ATR
515 (108). We and others have shown HPV8-associated impaired DNA repair, which would
516 facilitate the acquisition of transforming mutations (40, 109, 110).

517

518 HPV8 reactivation associated JZSC proliferation and expansion mirrors the pathological
519 findings in human AK (38). While the presence of koilocytes in AK has been reported, their
520 presence has previously been attributed to UV-induced transformation. Here we show that
521 the presence of koilocytes in AK is indicative of HPV8, with loss of Rb1 and increased
522 STAT3 phosphorylation. Although HPV8 E7 demonstrated lower binding of Rb1 and does
523 not directly cause degradation, we have previously shown reduced Rb1 levels in human
524 keratinocytes expressing E7 and all the complete early region genes (111–113). The
525 archetypal AK pathology findings include a dilated HF infundibulum with overlying
526 orthokeratosis and an accumulation of atypical keratinocytes within the perifollicular
527 epidermis (114). As would be expected from viral reactivation, AK are frequently observed

528 with a dense inflammatory cell infiltrate. While in our mouse models constitutive expression
529 of the HPV8 early region genes results in JZSC proliferation and expansion into the
530 overlying interfollicular epidermis, the ensuing immune response is able to restore
531 equilibrium in native infection. Thus, explaining the increased risk of AK in immune-
532 suppressed individuals and similarly why in otherwise healthy individuals, 87% of AK
533 spontaneously resolve within four-years (115). Herein we hypothesize that JZSC
534 proliferation and expansion into the overlying interfollicular epidermis occurs in the absence
535 of melanin protection, such that these keratinocytes easily accrue UV-induced mutations;
536 thus, providing a basis for the “hit and run” mechanism. Consistent with this we observed
537 p21 and p53 clones throughout the AK epidermis, and H2AX phosphorylation. In
538 conclusion, our findings in the context of HPV8 reactivation redefine human AK as a HF
539 disorder of KSCs and provides a mechanistic explanation for the ‘hit and run’ hypothesis for
540 HPV8 induced cSCC.

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551 **Methods**

552 Further information can be found in Supplemental Methods

553

554 **Sex as a biological variable**

555 For both human and animal models in this study, male and female samples were used, and
556 similar findings were reported for both sexes.

557

558 **Experimental Models**

559 **Mice**

560 B6.129P2-*Gt(ROSA)26Sor^{tm1(CAG-Brainbow2.1)Cle}/J*, (44, 116) *Lrig1^{tm1.1(cre/ERT2)Rjc}/J*, (117)

561 B6;SJL-Tg(Krt1-15-cre/PGR*)22Cot/J(118) were purchased from the Jackson Laboratory.

562 *Lrig1-EGFP-ires-CreER^{T2}* mice were a kind gift from Kim Jensen (University of

563 Copenhagen, Denmark)(46). *Krt14-HPV8-CER*,(39) *Krt14-HPV8-E2*,(42) *Krt14-HPV8-*

564 *E6*,(43) and *Krt14-HPV8-E7*(41) mice were used in this study. B6.129P2-

565 *Gt(ROSA)26Sor^{tm1(CAG-Brainbow2.1)Cle}/J*, *Lrig1^{tm1.1(cre/ERT2)Rjc}/J*, B6;SJL-Tg(Krt1-15-

566 *cre/PGR*)22Cot/J* and *Lrig1-EGFP-ires-CreER^{T2}* mice were backcrossed with FVBN mice

567 for six generations to yield a pure FVBN background and finally interbred with *Krt14-HPV8-*

568 *CER* and *Krt14-HPV8-E6* mice. *Stat3^{WT/LoxP}/FVBN* and *Stat3^{WT/WT}/FVBN* mice were crossed

569 with *Krt14-HPV8-CER* mice to generate *Stat3^{WT/LoxP}/Krt14-HPV8/FVBN* and

570 *Stat3^{WT/WT}/Krt14-HPV8/FVBN* mice.

571

572 **Tamoxifen and RU486 injection**

573 Cre activation in *Lrig1CreER^{T2}* mice was induced by injecting 4-week-old mice

574 intraperitoneally with 80 mg/kg/day of tamoxifen in corn oil for four consecutive days. Cre

575 activation in *Krt15CrePGR* mice was induced by injecting 4-week-old mice intraperitoneally

576 with 80 mg/kg/day of RU486 in corn oil for four consecutive days. Mice were harvested 30
577 days post induction.

578

579 **Cell lines**

580 Three established cell lines; HaCaT, PM1 and J2-3T3 were used in this research. Details
581 on cell culture conditions used can be found in the Supplemental Methods.

582

583 **Methods Details**

584 **Generation of transduced HaCaT and PM1 cell lines**

585 The Moloney murine leukemia retrovirus vector pLXSN (vector control) was used to
586 generate recombinant retroviruses containing HPV8 genes coding for HPV8 E6 and E7.
587 Briefly, retroviral transduction of HaCaT and PM1 cell lines were performed by seeding cells
588 into 6cm dishes, allowed to adhere overnight, before adding a mixture of retroviral
589 supernatants with an equal volume of DMEM in the presence of 5 µg/mL of hexadimethrine
590 bromide (polybrene). Spin infection was made by centrifugation for 1 hour at 300xg. Cells
591 were washed with PBS and cultured for two days before being selected for using G418 at a
592 concentration of 500 µg/mL.

593

594 **Tissue dissociation and culture**

595 Mouse dorsal back skin was dissociated into single cells as described previously (38).

596

597 **Murine primary colony forming assay culture**

598 Mouse dorsal back skin was dissociated into single cells as described above. Lrig1
599 expressing mouse keratinocytes were isolated through flow sorting and 2,500 cells were
600 seeded on an irradiated J2-3T3 feeder layer per well in a 6-well plate and cultured in

601 Rheinwald and Green media for 15 days, with media changed every 3 days. The colonies
602 were stained with crystal violet, scanned with a GelCount machine (Oxford Optronix,
603 Abingdon, UK) and quantified using ImageJ software (NIH, Bethesda, MD).

604

605 **Short interfering RNA knockdown experiments**

606 siRNA transfections were performed 24 hours after seeding keratinocytes with siRNA (ON-
607 Targetplus SMARTpool, Dharmacon), Lipofectamine 3000 transfection reagent
608 (ThermoFisher Scientific) and Opti-MEM (ThermoFisher Scientific). siRNA concentrations
609 were optimized individually. Cell lines were transfected with siRNA targeting STAT3
610 (20 η M), P300 (30 η M), YAP (20 η M).

611

612 **Calcium shift experiment on established cell lines**

613 Keratinocytes were de-differentiated by culturing cells for 48 hours in Epilife media
614 (containing no calcium chloride; MEPICF500). Keratinocytes were induced to differentiate
615 by adding Epilife media (containing 60 μ m calcium final concentration) and left for the
616 number of days stated in the figure to assess differentiation levels.

617

618 **Whole mount skin preparation and fluorescence imaging**

619 Tail skin was cut into 0.5cm² pieces and placed overnight at 4°C in Dispase (2.5U/mL).
620 Epidermis was gently removed from the underlying dermis using forceps and fixed in 10%
621 neutral buffered formalin for 90 minutes at room temperature. Tissue was washed in PBS
622 and stored in PBS+0.2% sodium azide at 4°C ready for immunofluorescence labelling.
623 Immunofluorescence on tail skin was performed as described previously (38). Antibodies
624 used can be found in Supplemental Table 5.

625

626 **Immunofluorescence staining and microscopy of OCT sections**

627 Immunofluorescence was performed on either frozen OCT embedded or paraffin embedded
628 sections as previously described (38). Further experimental details and antibodies used can
629 be found in the Supplemental Methods and Supplemental Table 5, respectively.

630

631 **Immunohistochemistry staining**

632 Rehydration of sections and antigen retrieval was performed as described in the
633 immunofluorescence staining above. Further experimental details and antibodies used can
634 be found in the Supplemental Methods and Supplemental Table 5, respectively.

635

636 **Starry-Warthin stain**

637 All reaction solutions were reduced from pH 4 to pH 3.2 before conducting the staining as
638 per manufacturer's instructions (Abcam, UK).

639

640 **Fluorescence-activated cell sorting (FACS) or analysis**

641 Samples were analyzed and flow sorted using BD LSR Fortessa and BD FACSAria Fusion
642 (BD Biosciences), respectively. Mouse telogen dorsal back skin was dissociated and
643 washed with FACS buffer (0.05% sodium azide and 0.5% BSA in PBS) before primary
644 antibody staining for 30 minutes on ice. Primary antibodies used in this study: Lrig1 488
645 (R&D systems, FAB3688G), Lrig1 647 (VWR, 10330-520), CD34 PE (BD, 551387).

646 Unbound antibodies were removed by washing with FACS buffer twice by centrifugation. All
647 centrifugations were performed at 250×g for 5 minutes at 4°C. Details on the gating
648 strategy can be found in the Supplemental Methods

649

650 **Western immunoblotting**

651 Whole protein lysate was extracted using Lysing Matrix D tubes (MP Biomedicals) by
652 homogenization for tissue or agitation with a pipette for cell pellets in RIPA buffer
653 (ThermoFisher Scientific) supplemented with 1x protease/phosphatase inhibitor cocktail
654 (Cell Signalling). Nuclear protein lysate was extracted using NE-PER™ Nuclear and
655 Cytoplasmic Extraction reagents (ThermoFisher Scientific). Details on how Western blotting
656 was performed and the antibodies used can be found in the Supplemental Methods and
657 Supplemental Table 5, respectively.

658

659 **Co-Immunoprecipitation**

660 Nuclear protein lysates were prepared from HaCaT cells at a confluency of 50-60%. Co-IP
661 experiments were performed using the Pierce™ Co-Immunoprecipitation kit (ThermoFisher
662 Scientific). Further details on how Co-Immunoprecipitation was performed can be found in
663 the Supplemental Methods.

664

665 **Chromatin Immunoprecipitation (ChIP)-qPCR**

666 ChIP-qPCR experiment was performed using the High-Sensitivity ChIP kit (Abcam) as per
667 manufacturers guidelines. Further details on how ChIP-qPCR was performed can be found
668 in the Supplemental Methods.

669

670 **Colony forming ability (established cell lines)**

671 HaCaT/PM1-PLXSN, -E6 and -E7 cells were seeded at a low density of 500 cells/well in a
672 6-well plate and left for 7 days in growth media to allow colonies to form. Colonies were
673 quantified by removing the media, washing with PBS and staining with crystal violet solution

674 for 15 minutes on a rocker at room temperature, before washing off solution by gently
675 running the plates under tap water. Plates were scanned and enumerated using a GelCount
676 plate reader (Oxford Optronix).

677

678 **RNA extraction and cDNA synthesis**

679 Depending on cell numbers, RNA was isolated using the Qiagen RNeasy Plus Mini or Micro
680 Kits (Qiagen, UK) per manufacturer's instructions. The quality of the extracted RNA was
681 assessed using the Agilent RNA 6000 Nano kit. Agilent Nano chips were run on the
682 Agilent 2100 Bioanalyzer according to manufacturer's guidelines. cDNA synthesis was
683 performed using the Quantitect Reverse Transcription Kit (Qiagen, UK) in 0.2 mL PCR
684 tubes as per manufacturer's instructions.

685

686 **Quantitative real-time PCR (qPCR)**

687 For qPCR gene expression studies, reactions were performed using TaqMan gene
688 expression probes. Pre-designed TaqMan primer/probes were obtained from Applied
689 Biosystems (see Supplemental Table 5). Reactions were run using the TaqMan Universal
690 Master Mix II (Applied Biosystems) according to the manufacturer's guidelines.

691 Housekeeping genes (GAPDH and β -actin) were used as reference genes. All reactions
692 were run in three technical triplicates, and all experiments were performed at least three
693 times independently. All reactions were run on the QuantStudio 7 Flex Real-Time PCR
694 system (Applied Biosystems) supplemented with the QuantStudio software. Gene
695 expression analysis of qPCR data was analyzed using the $\Delta\Delta C_t$ method to calculate fold
696 change ($2^{\Delta\Delta C_t}$) relative to control.

697

698 **DNA extraction, precipitation and β -HPV genotyping PCR**

699 DNA was extracted from 25mg of FFPE sections using the QIAamp DNA Mini Kit (Qiagen,
700 UK). For human samples with a DNA concentration $<10 \text{ ng}/\mu\text{L}$, DNA precipitation was
701 performed to gain a higher concentration and purity. To perform genotyping, the PM-PCR
702 Reverse Hybridisation Assay method was performed using RHA kit Stain (β) HPV kit (Labo
703 Bio-Medical Products BV). Details can be found in the Supplemental Methods.

704

705 **Nested PCR for HPV8 E6**

706 The molecular detection of HPV8 in the skin tissue samples was performed using nested-
707 PCR amplification. DNA was extracted from 44 FFPE samples exhibiting koilocytes and 33
708 FFPE samples with no koilocytes. Two sets of primers were designed, outer and nested.
709 Both outer and nested sets were flanking an area in E7-E1 region of HPV8. Details on
710 primer sequences and PCR reaction specifications can be found in the Supplemental
711 Methods.

712

713 **RNA sequencing of mouse dorsal back skin samples**

714 RNA for RNA-seq was extracted and RNA quality was assessed as mentioned previously.
715 Upper hair follicle (Lrig1) and bulge (CD34) stem cells were isolated by flow sorting from
716 telogen dorsal back skin in 7-week-old Krt14-HPV8-CER and WT mice. Twelve samples (3x
717 Lrig1+ WT, 3x Cd34+ WT, 3x Lrig1+ Krt14-HPV8-CER, 3x Cd34+ Krt14-HPV8-CER) were
718 sequenced (GSE248056) by Wales Gene Park (WGP). Confetti-positive cells with and
719 without Lrig1 cell surface expression were flow sorted from
720 Lrig1CreER^{T2}:R26RConfetti:HPV8-E6tg and Lrig1CreER^{T2}:R26RConfetti:WT mice. Twelve
721 samples (3x HPV8-E6tg Lrig1⁻Confetti⁺, 3x HPV8-E6tg Lrig1⁺Confetti⁺, 3x WT Lrig1⁻
722 Confetti⁺, 3x WT Lrig1⁺Confetti⁺) were sequenced (GSE248056). Total RNA was extracted
723 using an RNeasy Micro Kit (Qiagen). RNA was then frozen at -80°C and shipped to

724 Novogene (Cambridge, UK) on dry-ice for library preparation and sequencing. Further
725 details on bioinformatic analyses can be found in the Supplemental Methods.

726

727 **QUANTIFICATION AND STATISTICAL ANALYSIS**

728 **Statistics and reproducibility**

729 Statistical analyses were performed in GraphPad Prism v9. Data are presented as mean \pm
730 SEM. Two-tailed Student's t-test was used to measure significance between two groups,
731 while one-way ANOVA was used when comparing multiple groups. Specifically, statistical
732 tests applied for each figure can be found in Supplemental Information. p-values <0.05 are
733 considered significant. Symbols for significance: ns, non-significant, *, <0.05 ; **, <0.01 ; ***,
734 <0.001 ; ****, <0.0001 . For each experiment, n represents the number of experimental
735 replicates. For animal experiments, age- and sex-matched mice were randomly assigned to
736 groups, and at least three biological replicates were used for each experiment.

737

738 **Identification of koilocyte-like keratinocytes**

739 Human FFPE AK tissues (n=77) were serially sectioned with the first section stained with
740 H&E and imaged. Each image was then carefully assessed for the presence of koilocytes in
741 the epidermis. A sample was determined to be positive if multiple koilocytes were observed
742 within 100 μm of epidermis as single entities or clusters.

743

744 **Quantifying positively stained tissue sections**

745 To determine positive cell expression, images were analysed using Qupath software (119).
746 For pp53, p63, pSTAT3, p21, Rb1, p16 and pH2AX, the number of cells were determined
747 using the automated cell detection tool; nuclei with either a haematoxylin or DAB optical

748 density over the defined intensity threshold were counted and those with a DAB value over
749 the pre-determined positive threshold value defined as positive cells.

750

751 **Study Approval**

752 *Animals.* All mouse experiments carried out in this study were performed in accordance with
753 a UK Home Office Licence (project license 30/3382).

754

755 *Patient samples.* Human tissue samples were obtained after informed written consent from
756 patients following NHS Research and Development and Regional Ethics Committee
757 approval (19/NS/0012). Pathologist diagnosed actinic keratosis samples together with
758 anonymised clinical reports were collected. 275 patient samples were analyzed for the
759 presence of koilocytes by histology, with a prospective cohort of the first 77 samples further
760 studied in more detail for the presence of HPV8 via genotyping.

761

762 **Data and code availability**

763 RNA sequencing data have been deposited at GEO and are publicly available from the date
764 of publication. Accession number is GSE248056. This paper does not report original code.
765 Data are available in the “Supporting data values” XLS file. The lead contact can provide
766 any additional information required to reanalyze the data within this paper.

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772 **Author contributions**

773 GKP, BA, and MG conceived and supervised this study. GKP, HJM, CO, and BYS designed
774 the experiments. HJM and CO performed *in vitro* and protein experiments. HJM, CO, and
775 BYS performed *in vivo* experiments. AG, HJM, and CO performed bioinformatic analyses
776 on RNA-seq datasets. HJM, CO, AG, ALP, LA, and AL performed work on human AK
777 samples; LM and CB performed *in vivo* experiments and mouse tissue processing. MDA,
778 MH, BA, and MG contributed new reagents and expertise throughout the project; GKP and
779 RG conducted the human study; HJM, CO, BYS analyzed the data; GKP, HJM and CO
780 wrote the manuscript. All authors edited the manuscript. The order of co-first authorship
781 was determined by effort in data analysis and drafting the manuscript.

782

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790 Denmark) for providing the Lrig1-EGFP-ires-CreER^{T2} mouse model, and Sigrun Smola
791 (Institute of Virology, Center of Human and Molecular Biology Building, Saarland University
792 D-66421 Homburg Saar, Germany) for the transduced PM1 cell lines. We thank Dr Majid
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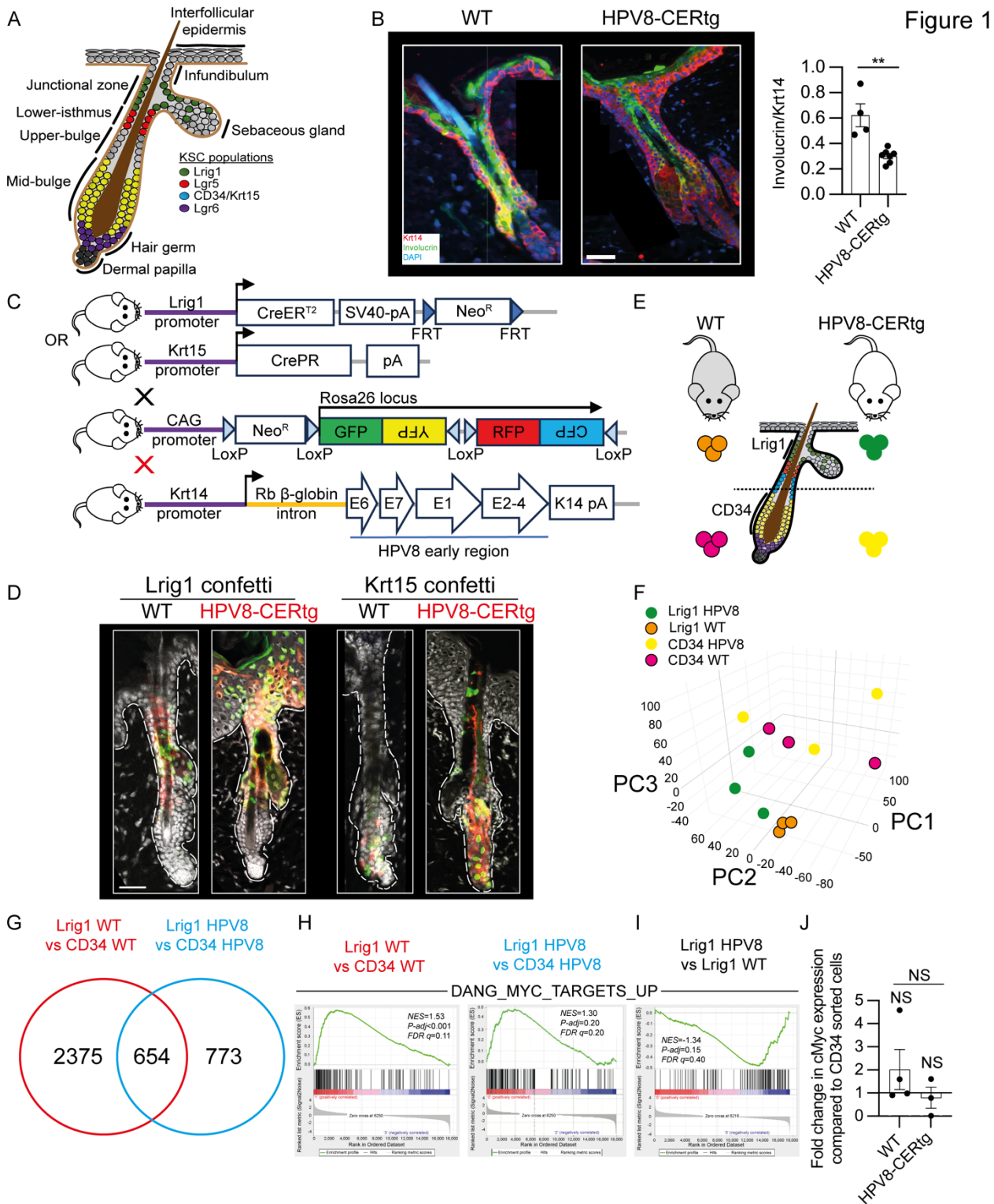
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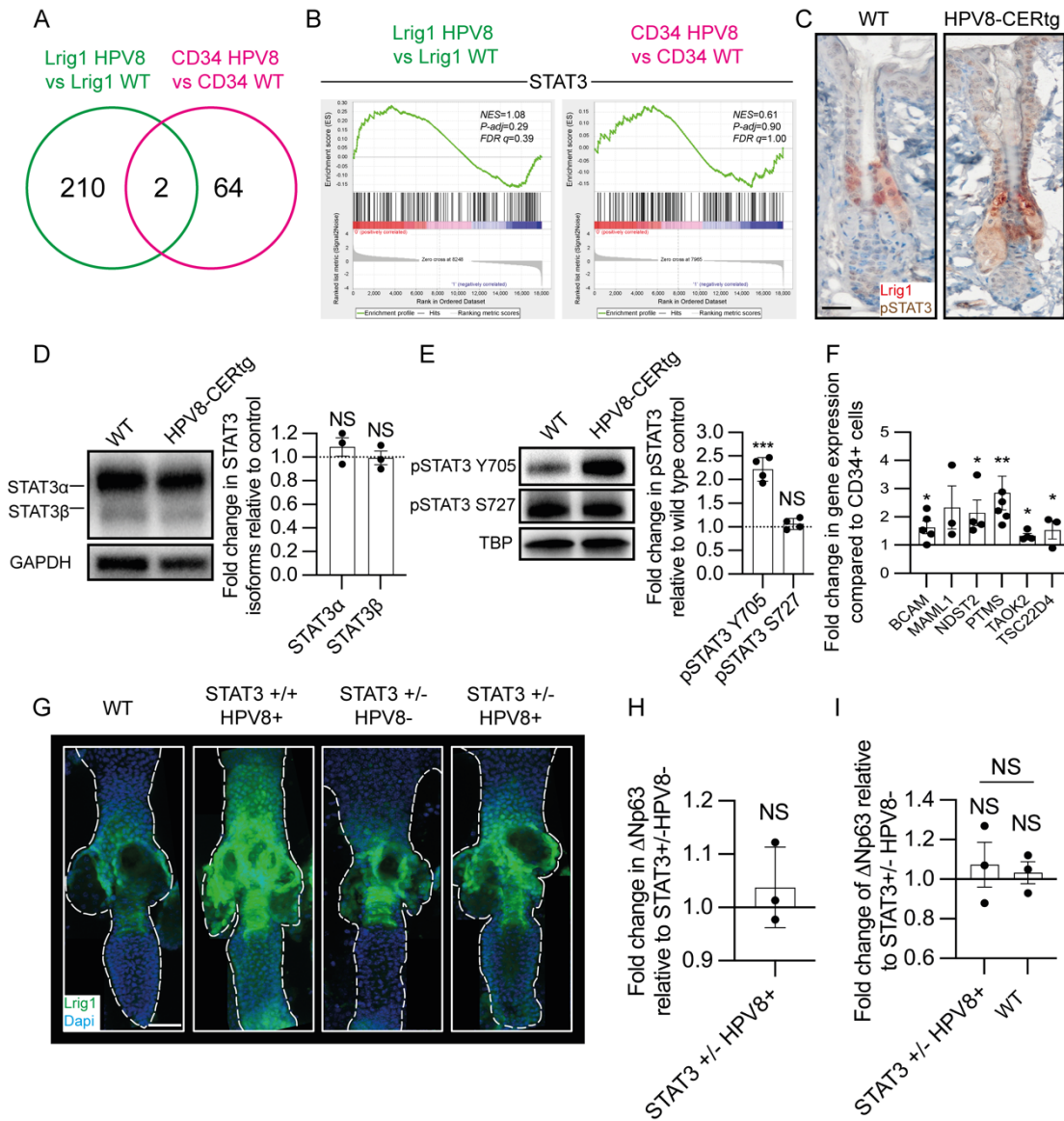
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Figure 1. HPV8 induced Lrig1⁺ hair follicle junctional zone KSC proliferation and expansion. (A) Schematic of hair follicle KSC populations. (B) Immunofluorescent labeling of WT (left) and HPV8-CERTg (right) adult back skin for involucrin (green) and keratin 14 (red), n=11 mice (average of 10 hair follicles/mouse). (C) Schematic summary of four mouse lines that were crossed for lineage tracing. (D) CLSM images of Lrig1 (left) and Keratin 15 (right) promoter-driven confetti reporter expression in progeny of WT (left) and HPV8-CERTg (right) adult back skin. (E) Experimental strategy for flow-sorting Lrig1⁺ and CD34⁺ populations for within-mouse comparisons. See also Supplemental Figure 1. (F) PCA of RNA-seq transcriptome analysis of skin KSC populations. (G) Venn diagram showing shared DEGs from Lrig1 vs CD34 comparisons (see Supplemental Table 1). (H) GSEA for c-Myc-regulated genes in DEGs from transcriptomic analysis. (I) GSEA for c-Myc-regulated genes in DEGs from Lrig1 flow sorted HPV8-CERTg vs WT transcriptomic analysis. (J) QPCR of RNA from flow-sorted cell isolated as in (E). All scale bars = 40µm. See also Supplemental Figure 1. Statistical test(s) Figure 1B 2-tailed Student's t-test, Figure 1J one-way ANOVA. **P<0.01.

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Figure 2



L097 **Figure 2. Activated STAT3 regulatory node in HPV8 in Lrig1⁺ hair follicle junctional zone KSC.** (A) Venn diagram showing shared
L098 DEGs from HPV8-CERTg vs WT KSC comparisons. (B) GSEA for STAT3-regulated genes in DEGs from transcriptomic analysis. See also
L099 Supplemental Figure 2. (C) IHC for pSTAT3 on adult back skin from WT and HPV8-CERTg mice. (D) Immunoblot of total STAT3 (α and β
L100 isoforms) in WT and HPV8-CERTg of adult back skin epidermal sheet extracts (n=3). Dotted line is the comparator. (E) Immunoblot of
L101 pSTAT3 Y705 and S727 in WT and HPV8-CERTg of adult back skin epidermal sheet nuclear extracts (n=4). Dotted line is the comparator.
L102 (F) QPCR of RNA from flow-sorted cell isolates as in (1E) for STAT3 downstream target genes (n \geq 3). (G) CLSM of whole mount tail skins
L103 from WT, HPV8-CERTg, STAT3^{-/-}, and STAT3^{+/-} HPV8-CERTg mice for Lrig1 (green) with DAPI (blue). (H) QPCR of RNA from STAT3^{+/-}
L104 and STAT3^{+/-} HPV8-CERTg adult back skin epidermal sheets for Δ Np63 (n=3). (I) QPCR of RNA from WT, STAT3^{+/-} and STAT3^{+/-} HPV8-
L105 CERTg flow sorted Lrig1⁺ KSC cell for Δ Np63 (n=3). All scale bars = 40 μ m. Statistical test(s) Figure 2D, E, F and H 2-tailed Student's t-
L106 test, Figure 2I one-way ANOVA. *P<0.05; **P<0.01; ***P<0.001.

Figure 3

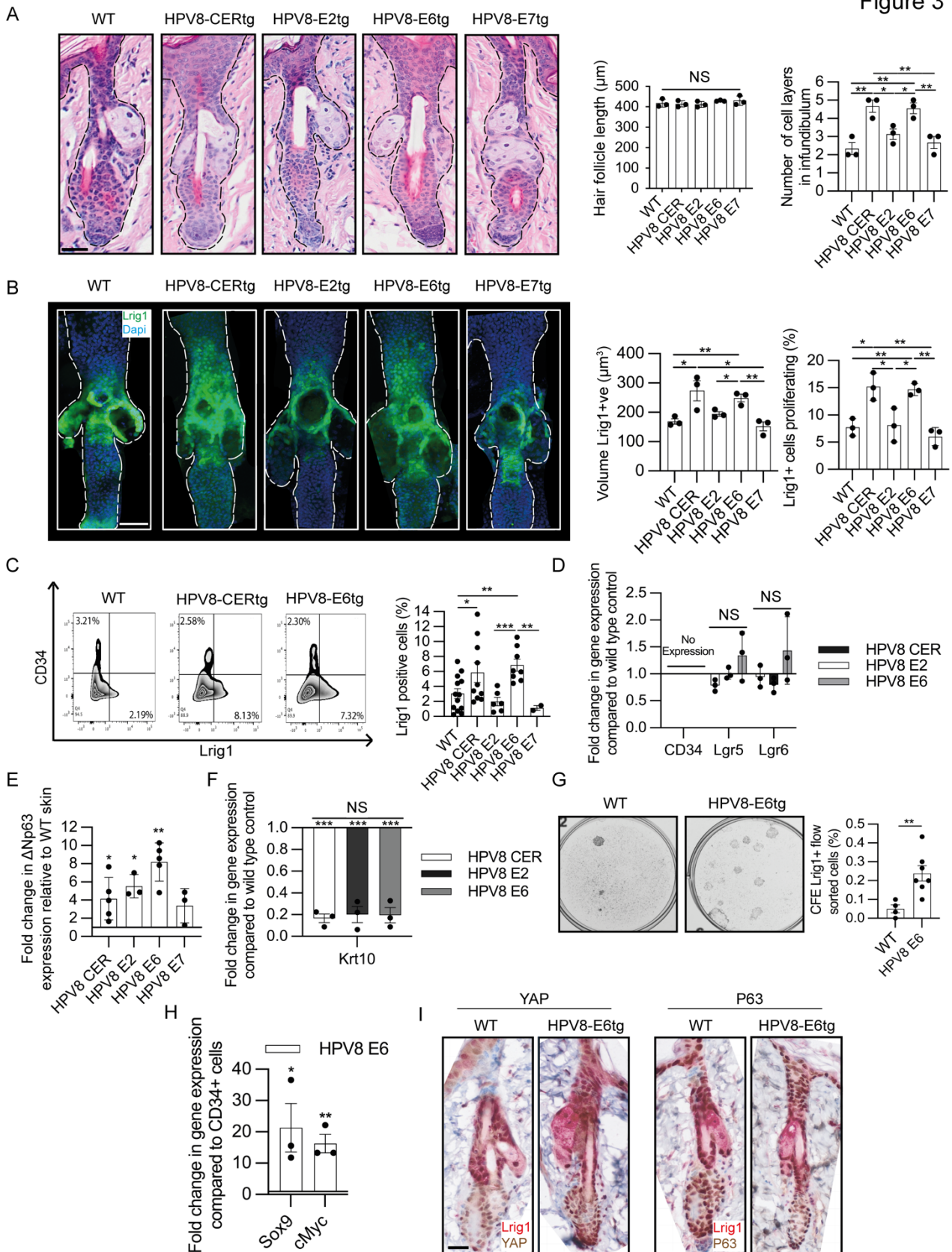


Figure 3. HPV8 E6 drives Lrig1⁺ hair follicle junctional zone KSC proliferation and expansion. (A) Haematoxylin and Eosin-stained sections from WT, HPV8-CERtg, HPV8-E2tg, HPV8-E6tg, and HPV8-E7tg adult back skin, with quantification of hair follicle length and number of cell layers in the infundibulum (n=3 mice/genotype, average of 20-50 hair follicles/mouse). (B) CLSM of whole mount tail skins as in (A) labeled for Lrig1, with quantification of Lrig1 labeled volume and the number of co-labeled Ki67+ cells (average of 10 hair follicles/mouse). (C) FACS for Lrig1 and CD34 positive populations from back skin cell isolates as in (A) (n=39 total). (D) QPCR of RNA from Lrig1⁺ flow-sorted cell isolates as in (A) for KSC markers (n=3). (E) QPCR of RNA from Lrig1⁺ flow-sorted cell isolates as in (A) for ΔNp63 (n=20 total). (F) QPCR of RNA from Lrig1⁺ flow-sorted cell isolates as in (A) for keratin 10 (n=3). (G) CFE of 2500 flow-sorted Lrig1⁺ keratinocytes from WT and HPV8-E6tg adult back skin epidermal sheets (n=11 total). (H) QPCR of RNA from Lrig1⁺ and CD34⁺ flow-sorted cell isolates from HPV8-E6tg adult back skin epidermal sheets (n=3). (I) IHC for Lrig1 together with YAP (left) or p63 (right) on WT and HPV8-E6tg adult back skin. All scale bars = 40 μm . See also Supplemental Figure 2. Statistical test(s) Figure 3A, B, C, D, E and F one-way ANOVA, Figure 3G and H 2-tailed Student's t-test. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

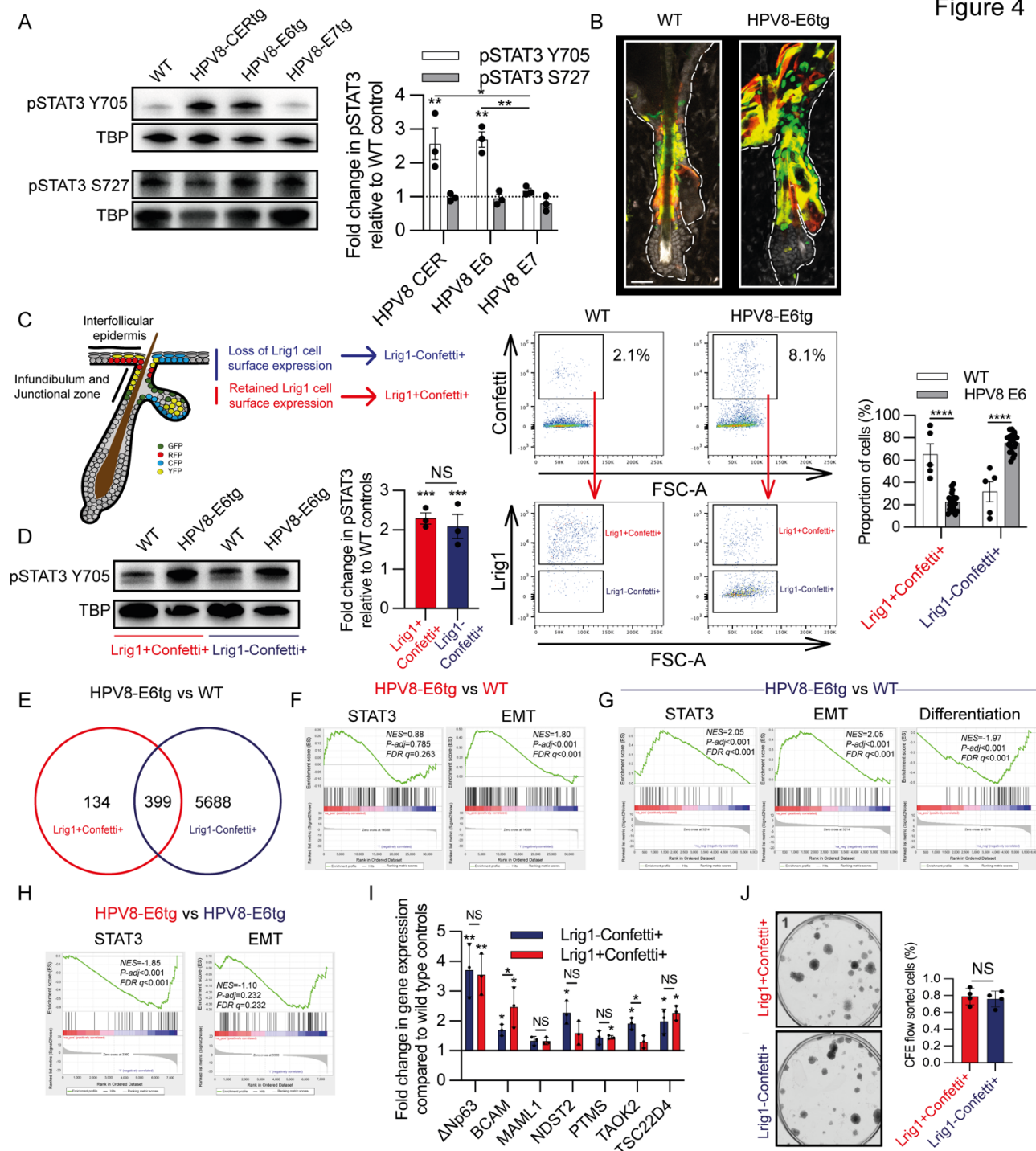
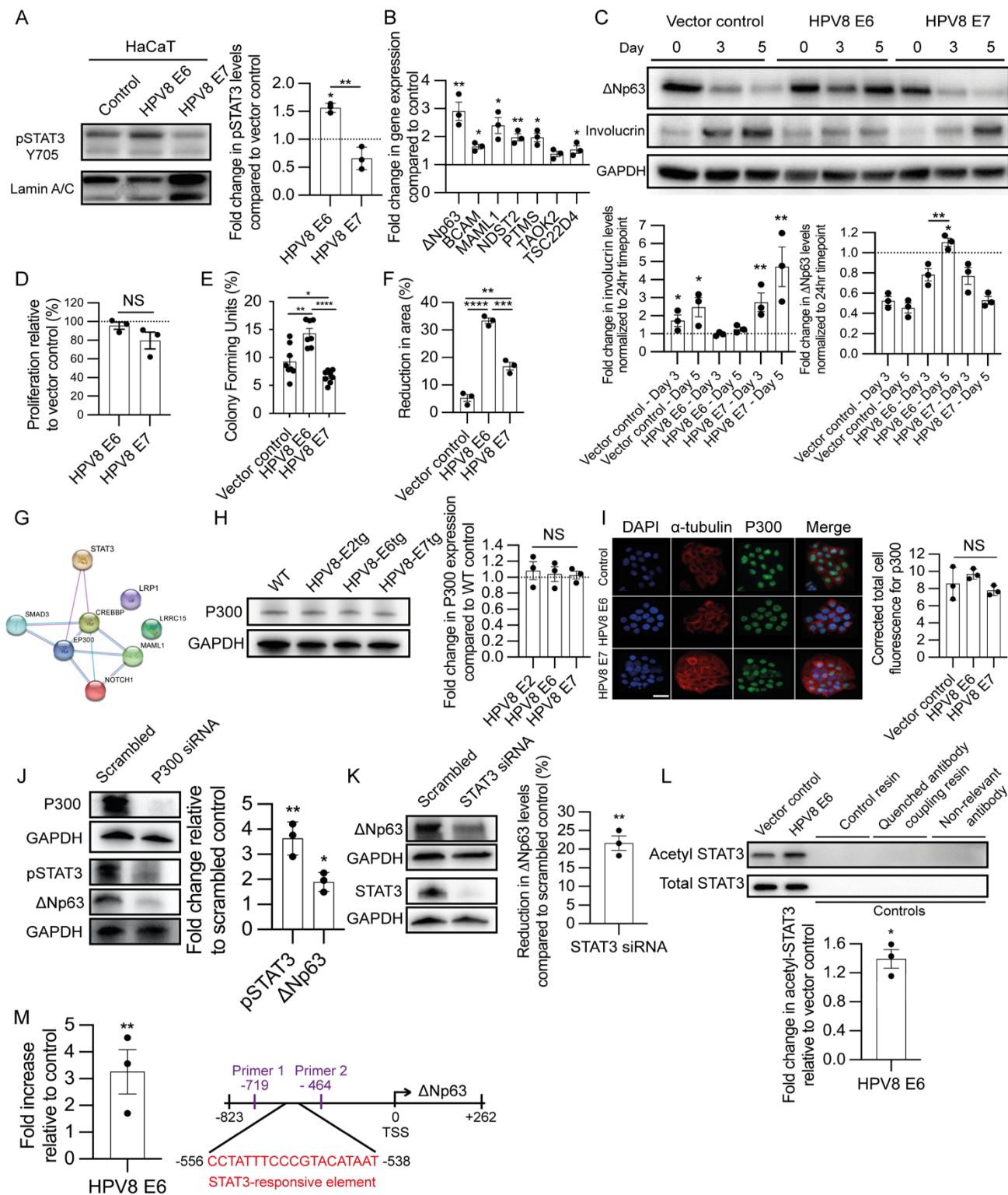


Figure 4. Lig1⁺ hair follicle junctional zone KSC progeny retain KSCs. (A) Immunoblot of pSTAT3 Y705 and S727, with TATA-Box binding protein (TBP) control (n=3). (B) CLSM of dorsal back skin for lineage tracing of Lig1CreER^{T2}:R26RConfetti:WT and Lig1CreER^{T2}:R26RConfetti:HPV8-E6tg progeny, after 4-weeks post Cre activation. Scale bar = 40μm. (C) Enumerated Lig1⁺ confetti⁺ and their progeny Lig1⁻ confetti⁺ flow-sorted cell populations from Lig1CreER^{T2}:R26RConfetti:HPV8-E6tg and Lig1CreER^{T2}:R26RConfetti:WT mice (n=25 total). (D) Immunoblot of Lig1⁺ confetti⁺ and their progeny Lig1⁻ confetti⁺ flow-sorted cell populations (n=3). (E) Venn diagram showing shared DEGs from Confetti HPV8 E6 vs Confetti WT comparisons for Lig1⁺ confetti⁺ and Lig1⁻ confetti⁺ populations (see Supplemental Table 2). (F) GSEA for STAT3- and EMT-associated gene signatures in DEGs from Lig1⁺ confetti⁺ transcriptomic comparison of Confetti HPV8 E6 vs Confetti WT analysis. (G) GSEA for STAT3-, EMT- and differentiation-associated gene signatures in DEGs from Lig1⁻ confetti⁺ transcriptomic comparison of Confetti HPV8 E6 vs Confetti WT analysis. (H) GSEA for STAT3- and EMT-associated gene signatures in DEGs from Confetti HPV8 E6 transcriptomic comparison of Lig1⁺ confetti⁺ and Lig1⁻ confetti⁺ population analysis. See also Figure S3. (I) QPCR of RNA from flow-sorted cell isolates as in (C) for STAT3-regulated genes (n=3). (J) CFE of 2500 flow-sorted Lig1⁺ confetti⁺ and Lig1⁻ confetti⁺ flow-sorted cell populations from Lig1CreER^{T2}:R26RConfetti:HPV8-E6tg (n=4). See also Supplemental Figure 3. Statistical test(s) Figure 4A, D and I one-way ANOVA, Figure 4C and J 2-tailed Student's t-test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Figure 5



145 **Figure 5. HPV8 E6 P300 interaction activates STAT3.** (A) pSTAT3 Y705 immunoblot, with laminin A/C control, of nuclear extracts from transduced HaCaT keratinocytes (n=3). (B) qPCR of RNA from transduced HaCaT keratinocytes for STAT3-regulated genes, with b-actin as control (n=3). (C) Immunoblot of ΔNp63 and involucrin, with GAPDH as control, of transduced HaCaT keratinocytes cultured in high calcium (60 μm) media for 3 and 5 days (n=3). Proliferation (n=3) (D), CFE assay (n=7) (E), and migration (n=3) (F) of transduced HaCaT keratinocytes. (G) String™ analysis demonstrating the interaction of known HPV8 E6 protein binding partners and STAT3. Line colours define interactions as experimentally determined (pink) or from curated database (blue). (H) P300 with GAPDH control immunoblot of WT and HPV8-E2tg, -E6tg and -E7tg mouse keratinocytes (n=3/genotype). (I) P300, α-tubulin and DAPI immunofluorescence labeling of transduced HaCaT keratinocytes (n=3). Scale bar = 40mm. (J) P300, pSTAT3 Y705 and ΔNp63 immunoblot, with GAPDH endogenous control, of HPV8 E6 transduced HaCaT keratinocytes treated with scrambled control and p300 targeting siRNA (n=3). (K) STAT3 and ΔNp63 immunoblot, with GAPDH control, of HPV8 E6 transduced HaCaT keratinocytes treated with scrambled control and STAT3 targeting siRNA (n=3). (L) Immunoblot of STAT3 immunoprecipitated nuclear protein from vector and HPV8 E6 transduced HaCaT keratinocytes probed for acetylated STAT3 and total STAT3 (n=3). (M) qPCR analysis of ΔNp63 primers on STAT3 chromatin immunoprecipitants in HPV8 E6 transduced HaCaT keratinocytes relative to vector (n=3) Schematic of the 5'-flanking region indicating primers sequences relative to STAT3-RE and ΔNp63 TSS. See also Supplemental Figure 4. Statistical test(s) Figures 5A, C, D, E, F, H and I one-way ANOVA, Figure 5B, J, K, L and M 2-tailed Student's t-test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Figure 6

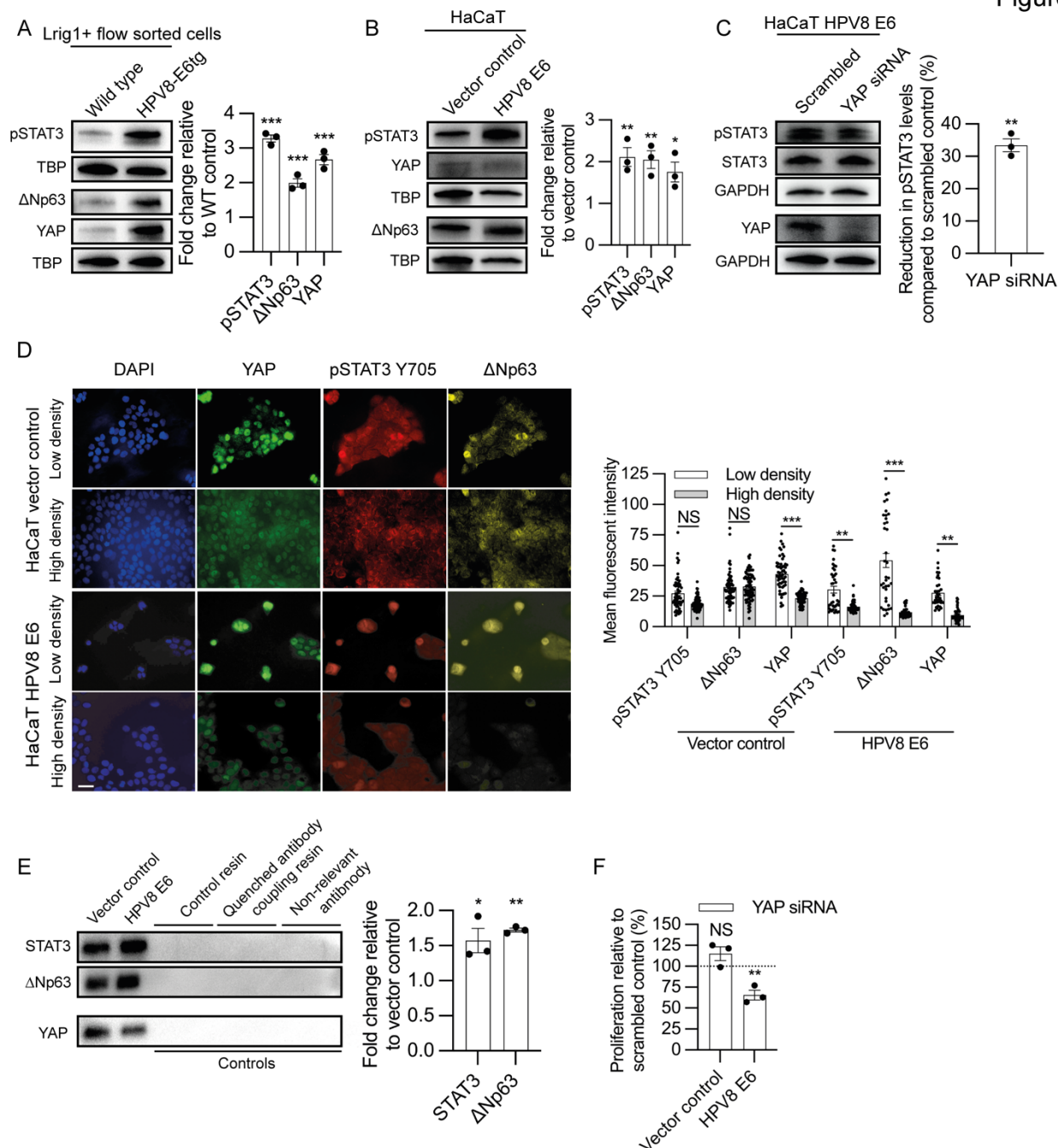


Figure 6. YAP a co-transcription factor for STAT3 and ΔNp63. (A and B) pSTAT3 Y705, ΔNp63 and YAP immunoblot, with TATA-box binding protein endogenous control, of nuclear extracts from Lrig1⁺ flow-sorted WT and HPV8-E6tg mouse keratinocytes (A) and HPV8 E6 and vector control transduced HaCaT keratinocytes (B) (n=3 per genotype/cell line). (C) pSTAT3 Y705, total STAT3 and YAP immunoblot, with GAPDH endogenous loading control, of HPV8 E6 transduced HaCaT keratinocytes treated with scrambled control and YAP targeting siRNA (n=3). (D) Immunofluorescent labeling of HPV8 E6 and vector control transduced HaCaT keratinocytes cultured at low (~50%) and high (~90%) confluency for YAP (green), pSTAT3 Y705 (red) and ΔNp63 (yellow), with quantification of nuclear mean fluorescent intensity (n=82 cells total quantified over three independent experiments). Scale bar = 40μm. (E) Immunoblot of YAP immunoprecipitated nuclear protein from vector control and HPV8 E6 transduced HaCaT probed for STAT3 and ΔNp63 (n=3). (F) Proliferation of HPV8 E6 and vector control transduced HaCaT keratinocytes assessed by 24 hours of BrdU incorporation following treatment with YAP targeting siRNA (n=3). See also Supplemental Figure 5. Statistical test(s) Figure 6A, B, C, D and E 2-tailed Student's t-test, Figure 6F one-way ANOVA. *P<0.05; **P<0.01; ***P<0.001.

Figure 7

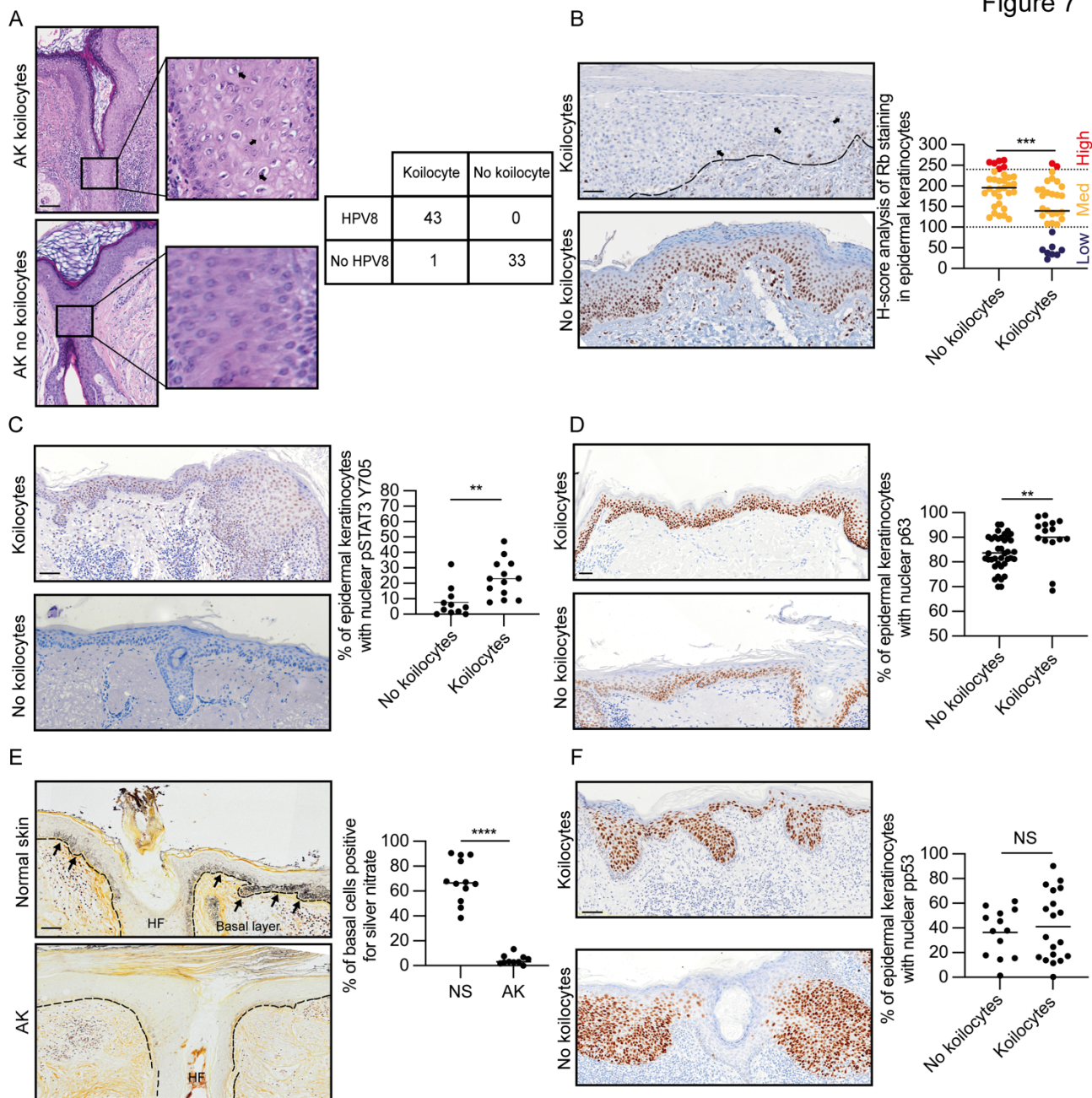


Figure 7. HPV8 reactivation in actinic keratosis with koilocytes. (A) (Left) Haematoxylin and Eosin-stained human AK sections with and without koilocytes. (Right) Presence of koilocytes by HPV8 detection using β -HPV L1 open reading frame PCR-reverse hybridisation assay. See also Supplemental Figure 6A. Arrows indicate the presence of koilocytes. (B-D) IHC of human AK tissue for Rb (n=64), pSTAT3 Y705 (n=24) and p63 (n=53). Arrows indicate the presence of koilocytes. (E) Warthin-Starry stain of human AK tissue (n=24). (F) IHC of human AK tissue for p53 (n=32). All scale bars = 50 μ m. See also Supplemental Figure 6. Statistical test(s) Figure 7B, C, D, E and F 2-tailed Student's t-test. ** P <0.01; *** P <0.001; **** P <0.0001.

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l185 **Supplemental Table 1. List of significant differentially expressed genes (adjusted p <**
l186 **0.05), Related to Figures 1, 2 and Supplemental Figure 1**

l187 For each gene, given are Log₂FC. Each tab represents a different comparison.

l188 **Supplemental Table 2. List of significant differentially expressed genes (adjusted p <**
l189 **0.05), Related to Figures 4 and Supplemental Figure 3**

l190 For each gene, given are Log₂FC. Each tab represents a different comparison.

l191 **Supplemental Table 3. List of activated 'Hallmark', 'Biocarta' and 'Wikipathways'**
l192 **pathways following gene set enrichment analysis on HPV8-E6tg vs WT**

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